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THE INACTIVATION OF PREGNANT MARES' SERUM GONADOTROPHIN BY HYDROQUINONES AND QUINONES

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ABSTRACT

Pregnant mares' serum (PMS) when incubated *in vitro* with hydroquinones and quinones loses its gonadotrophic activity. The influence of the incubation conditions on this process has been examined. Hydroquinones did not inactivate PMS unless they were first allowed to oxidize to the corresponding quinones. *p*-Benzoquinone was found to be more active than mono-substituted quinones and the di- and tetra-substituted quinones had little or no activity. The amorphous polymer "humic acid" formed by "ageing" *p*-benzoquinone solutions partially inactivated PMS but it was much less active than the parent compound.

IT WAS claimed by Sanyal (1) that 2:6-dimethylhydroquinone in the diet caused sterility in female rats, and he suggested that this effect was the result of interference with the peripheral action of progesterone. Noble and Graham (2) showed that pregnant mares' serum gonadotrophin (PMS) lost its biological activity when incubated with certain hydroquinones and quinones. They suggested that these compounds might be able to inactivate circulating gonadotrophins in the intact animal, thereby inhibiting the reproductive function.

Although the assay method used by Noble and Graham (2) revealed the antigonadotrophic action of hydroquinones and quinones *in vitro*, it was not sufficiently sensitive for the detection of small differences in activity between closely related compounds. The present work was undertaken

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[2] The data in this paper are taken in part from a thesis submitted by one of us (R.C.B.G.) to the Faculty of Graduate Studies, University of Western Ontario, in partial fulfillment of the requirements for the degree of doctor of philosophy.

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to determine more precisely the conditions under which these compounds inactivate PMS *in vitro* in order to obtain information on the structural features needed for antigonadotrophic activity, and on the mechanism of inactivation.

Hydroquinones are known to be oxidized to quinones by oxygen in slightly alkaline media (3, 4). In addition many quinones are themselves unstable in aqueous media, particularly if the latter are slightly alkaline, forming brown amorphous polymers termed "humic acids" (5, 6, 7). It was important, therefore, to establish whether the antigonadotrophic activity observed by Noble and Graham (2) was due to hydroquinones, quinones, "humic acids" or combinations of these substances.

METHODS

Immature female rats (3–5 weeks old) of the Sprague-Dawley strain were used. They were maintained on Master's Fox Breeder Starter and tap water *ad libitum*. The PMS[4] assayed at 45 I.U. per mg. The various hydroquinones and quinones were either recrystallized or sublimed at low temperature *in vacuo* immediately before use. The assay method was adapted from that described by Noble, Plunkett and Graham (8) for measuring the antigonadotrophic activity of Lithospermum extracts *in vitro*. For the present work the pH of the various media was carefully controlled. PMS was dissolved in phosphate buffer (0.067M) of the required pH to give a solution containing 500 I.U. per ml. One ml. of this solution was diluted to 2.5 ml. with a similarly buffered solution of the compound under test. The mixture was incubated at 37° C for varying periods in air, oxygen or nitrogen as indicated below. When working in nitrogen, precautions were taken to displace dissolved oxygen from all solutions with a stream of purified nitrogen. As far as possible the solutions were shielded from light to minimize photoxidation of the compounds. After incubation, aliquots of the solutions were administered to groups of rats by subcutaneous injection in the lateral area. Each animal received a single dose of 0.5 ml. of test solution containing either 100 I.U. of PMS (control), or 100 I.U. of PMS plus the substance under test. Three days later the animals were killed with chloroform anesthesia and the ovaries and uteri were carefully dissected out and weighed. Where a sufficient number of animals were used the organ weight data were analyzed by Fischer's "t" test (9).

RESULTS

Experiments with Hydroquinones and Related Compounds

Separate solutions of PMS and hydroquinones were prepared in phosphate buffer (0.067M) at pH 6.5, 7.5 and 8.0, and in borate buffer (0.067M) at pH 8.5. Parallel preparations were made under nitrogen, air and oxygen at each hydrogen ion concentration. Portions of these solutions were then mixed to give final concentrations of 200 I.U. of PMS and 0.2 mg. of hydroquinone per ml. After incubation at 37° C for 2 hours in the appropriate atmosphere, 0.5 ml. of each solution was injected into the test animals. The effect of incubating PMS with hydroquinone on the ovarian weights of the rats is shown in Figure 1.

[4] Kindly supplied by Ayerst, McKenna and Harrison Ltd., Montreal.

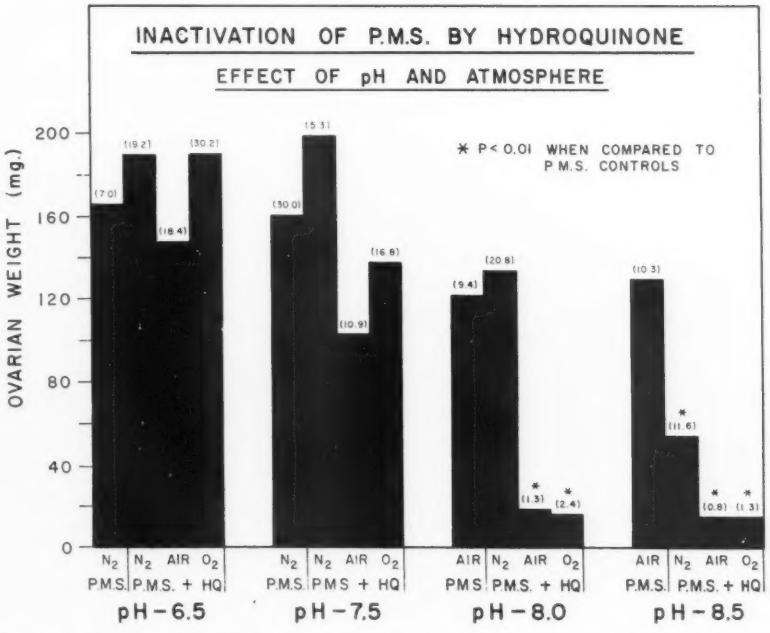

FIG. 1. *Inactivation of PMS by Hydroquinone.* 4 rats per group. HQ—hydroquinone. The number above each bar is the standard error of the mean. The experimental conditions are described in the text.

The results show that in the absence of oxygen (or air) hydroquinone did not inhibit PMS, except at pH 8.5 where there was partial inhibition. However, in the presence of oxygen the gonadotrophin was inactivated on incubation with solutions of hydroquinone if the pH of the medium was above 7.5, but not otherwise.

It may be noted in Figure 1 and in some of the following tables that there is a rather wide variation from group to group in the average ovarian weight of animals receiving PMS alone (i.e. of the control group). This is due mainly to (a) the partial loss of gonadotrophic activity which results when PMS alone is incubated in media of increasing alkalinity and (b) the influence of the age of the rats on the degree of response to PMS. Even though immature, the older rats responded to a greater degree than the younger ones. These experiments were performed at different times with rats of varying age (3–5 weeks), but in any one experiment with a PMS control the rats were approximately of the same age and the results can be compared with those of the corresponding control.

Phenylhydroquinone, methylhydroquinone, gallic acid, pyrogallol, gentisic acid, homogentisic acid, 3:4-dihydroxyphenylalanine (dopa), and rehibin ("trigentisic acid" (10)) were also incubated with PMS at pH 8.0

TABLE 1. ACTION OF SUBSTITUTED POLYPHENOLS ON THE GONADOTOPHIC ACTIVITY OF PMS

Compound	Dose per rat	Atmosphere during incubation	No. of rats	Ovarian wt. (mg.)
PMS control	100 i.u	air	4	115 ± 7.6
+HQ	0.1 mg.	air	4	18 ± 1.3**
+PHQ	0.1 mg.	N_2	4	125 ± 10.2
+PHQ	0.1 mg.	O_2	4	26 ± 4.0**
+PHQ	0.1 mg.	air	4	28 ± 2.6**
+PHQ	0.05 mg.	air	4	44 ± 1.0**
+PHQ	0.025 mg.	air	4	93 ± 2.5
+MHQ	0.5 mg.	N_2	4	118 ± 8.9
+MHQ	0.5 mg.	O_2	4	20 ± 0.8**
+MHQ	0.5 mg.	air	4	42 ± 3.4**
+MHQ	0.1 mg.	air	4	67 ± 8.2**
PMS control	100 i.u.	air	3	142 ± 25.2
+gallic acid	0.1 mg.	air	3	50 ± 5.6*
+pyrogallol	0.1 mg.	air	3	73 ± 15.2
+gentisic acid	0.1 mg.	air	3	123 ± 9.8
+homogentisic acid	0.1 mg.	air	4	82 ± 6.2
+dopa	0.1 mg.	air	4	145 ± 13.5
+rehibin#	0.1 mg.	air	2	132 ± 25.0

All incubations with PMS were made at pH 8.0 for 2 hours at 37° C.
HQ—hydroquinone.
PHQ—phenylhydroquinone.
MHQ—methylhydroquinone.
Ovarian weights are the mean ± S.E.M.
rehibin—"trigentisic acid" of Hahn (10).
** $P < 0.01$ when compared with PMS control.
* $P < 0.05$ when compared with PMS control.

for 2 hours at 37° C. The atmosphere used during the incubation, the dose level and the ovarian weights of the test animals are shown in Table 1.

Phenylhydroquinone and methylhydroquinone also failed to inactivate PMS at pH 8.0 in the *absence* of oxygen. Of the other related compounds only gallic acid showed significant activity when incubated with PMS in the presence of air. These findings suggested that the hydroquinones themselves were not inhibitors of the gonadotrophic activity of PMS, but formed inhibitors on oxidation in slightly alkaline media.

As shown in Figure 1, PMS was not significantly inactivated when incubated at pH 7.5 with hydroquinone. In order to distinguish between the effect of the pH of the medium on the formation of the presumed inhibitors and on their subsequent reaction with PMS, hydroquinone was pretreated with dilute alkali prior to its incubation at pH 7.5 with the gonadotrophin. An aqueous solution of hydroquinone was adjusted to pH 12 with dilute sodium hydroxide, and a portion was allowed to stand in air for 20 minutes at 20° C with occasional stirring. Another portion was saturated with oxygen by brisk passage of the gas through it for 5 minutes, stoppered and allowed to stand at 20° C for a further 40 minutes. Both solutions, which became dark brown during this treatment, were then adjusted to pH 7.5 by the cautious addition of dilute phosphoric acid. Suitable portions were incubated with PMS at pH 7.5 and 37° C for 2 hours in air. The dose

TABLE 2. EFFECT OF PRETREATMENT OF HYDROQUINONE PRIOR TO INCUBATION WITH PMS

Compound	Pretreatment at 20° C	Dose per rat#	Ovarian wt. (mg.)
PMS control	nil	100 i.u.	156 ± 31.2
+HQ	nil	0.1 mg.	103 ± 10.9
+HQ	NaOH-pH 12.0 Air for 20 mins.	0.1 mg.	52 ± 5.8*
+HQ	NaOH-pH 12.0 O_2 for 45 mins.	0.1 mg.	49 + 6.9*

All incubations with PMS were made at pH 7.5 for 2 hours in air at 37° C. (see text).
4 rats per group.
HQ—hydroquinone.
Ovarian weights are the mean ± S.E.M.
* $P < 0.02$ when compared PMS control.

given to the rats and their ovarian weights at autopsy are shown in Table 2. Control groups that received PMS incubated at pH 7.5, both alone and with freshly prepared hydroquinone are also included in this table.

Although solutions of hydroquinone were non-inhibitory to PMS when tested directly at pH 7.5, they became so at that pH if given a short pretreatment with dilute alkali in the presence of air. In aqueous alkaline solution, hydroquinones as a class are readily oxidized by molecular oxygen to the corresponding quinones. James, Snell and Weissberger (4) found that the reaction rate was closely dependent on the square of the hydroxyl ion concentration in the pH range 7.2 to 8.2. It therefore seems likely that in this pH range hydroquinone inactivated PMS in the presence of oxygen, because it first formed p-benzoquinone. The latter may have been the actual inhibitor or a precursor thereof. The next section deals with the reaction of p-benzoquinone with PMS.

Experiments With p-Benzoquinone

Effect of pH and atmosphere on inactivation of PMS

p-Benzoquinone was incubated for 2 hours at 37° C with PMS in buffered solutions at pH 6.5, 7.0, 7.5 and 8.0. Parallel experiments were made in most cases in an atmosphere of nitrogen or air. Each rat in the test received 0.5 ml. of the incubated solution containing initially 100 I.U. of PMS and 0.1 mg. of quinone. The ovarian and uterine weights of the rats at autopsy are shown in Figure 2.

From Figure 2 it may be seen that the ovaries of rats receiving PMS which had been incubated with *p*-benzoquinone at pH 7.0, 7.5 and 8.0 were small, irrespective of whether the incubation was in the absence or presence of oxygen. Partial inhibition of the gonadotrophic activity of PMS was found even at pH 6.5. At pH 8.0 the uteri were also significantly smaller than those of the PMS controls, indicating almost complete inhibition of the PMS. At pH 6.5 the uteri were larger than those of the PMS controls, the increased weight being due solely to fluid in the lumen. Histologically these uteri showed an enlarged fluid-filled lumen and thickened, regular endometrium, typical of a uterus stimulated by estrogen alone. The corresponding ovaries contained only a few large follicles; there

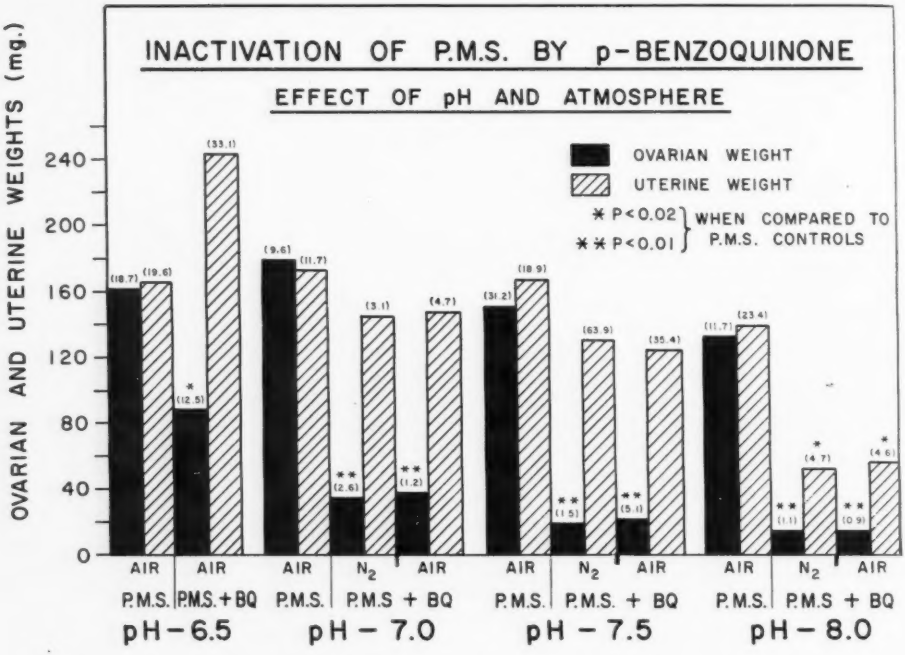

FIG. 2. *Inactivation of PMS by p-Benzoquinone.* 4 rats per group. BQ—p-benzoquinone. The number above each bar is the standard error of the mean. The experimental conditions are described in the text.

was some thecal luteinization, but no corpora lutea were seen. Stimulation of the interstitial cells was a prominent feature in these ovaries. Similar histological features were seen in ovaries and uteri from rats treated with PMS and "humic acid" (Table 5). Thus, slight inhibition of PMS produced large fluid-filled uteri, further inhibition produced uteri of the same weight (but containing fluid) as the PMS controls, and with complete inhibition the uteri were small and immature. These observations show that *p*-benzoquinone may either directly inactivate PMS or give rise—even in the absence of oxygen at pH 7.0—to products which do.

Relationship of quinone concentration to inactivation of PMS

The standard dose of PMS (100 I.U.) and varying amounts of *p*-benzoquinone were incubated together in air at 37° C for 2 hours. The incubations were done in phosphate buffer at pH 7.0 and 8.0. The quantities of *p*-benzoquinone used and the ovarian and uterine weights of the rats that received injections of these solutions are shown in Table 3.

The data in Table 3 show that the greater inhibition observed at pH 8.0 over that at pH 7 (Fig. 2) is maintained as the benzoquinone concentration was lowered. In the more alkaline medium both the ovaries and

TABLE 3. INACTIVATION OF PMS BY p-BENZOQUINONE: EFFECT OF QUINONE CONCENTRATION

Compound	Dose per rat#	pH	Ovarian wt. (mg.)	Uterine wt. (mg.)
PMS control	100 i.u.	7.0	180 ± 9.6	173 ± 11.7
+BQ	0.1 mg.	7.0	38 + 1.2**	151 ± 4.7
+BQ	0.05 mg.	7.0	73 ± 3.0**	163 ± 17.1
+BQ	0.025 mg.	7.0	110 ± 5.6**	166 ± 11.4
PMS control	100 i.u.	8.0	135 ± 11.7	140 ± 23.4
+BQ	0.1 mg.	8.0	16 ± 1.1**	43 ± 3.5*
+BQ	0.075 mg.	8.0	16 ± 0.9**	49 ± 4.7*
+BQ	0.05 mg.	8.0	20 ± 1.3**	52 ± 4.8*
+BQ	0.025 mg.	8.0	30 ± 2.0**	154 ± 25.8
+BQ	0.01 mg.	8.0	71 ± 3.0**	196 ± 40.0
+BQ	0.005 mg.	8.0	93 ± 9.6	153 ± 23.2

All incubations with PMS were made for 2 hours in air at 37° C.
4 rats per group.
BQ—p-benzoquinone.
Ovarian and uterine weights are the mean ± S.E.M.
** $P < 0.01$ when compared with PMS controls.
* $P < 0.02$ when compared with PMS controls.

the uteri were still significantly smaller than the PMS controls when the rats received 100 I.U. of PMS which had been incubated with 0.05 mg. of *p*-benzoquinone. Even 0.025 mg. of quinone at pH 8.0 inhibited the gonadotrophin sufficiently to prevent an increase in the ovarian weights, although the uteri now showed a secondary response.

Effect of time of incubation on inactivation of PMS

The standard dose of PMS was incubated with varying quantities of *p*-benzoquinone at 37° C in air at pH 8.0; the time of incubation ranged from 15 minutes to 24 hours. It was found that the inactivation of PMS by *p*-benzoquinone increased steadily as the time of incubation was extended from 15 minutes to 2 hours, with a slight further increase after 6 hours. Incubation beyond 6 hours did not cause further inactivation of the gonadotrophin. This raised the question of the effect of the longer incubation periods, not only on the reaction between the quinone and hormone, but also on the decomposition of the quinone itself. There are numerous references in the literature to the instability of quinones in aqueous media. In particular, these compounds frequently polymerize to form dark amorphous polymers known as quinone "humic acids," a reaction which proceeds in the absence of oxygen (4, 5, 6, 7, 11). Experiments related to this question are described below.

Experiments with Other Quinones

Phenylbenzoquinone, methylbenzoquinone, 2:5-dimethylbenzoquinone, and tetramethylbenzoquinone were incubated in the amounts shown in Table 4 with 100 I.U. of PMS. The incubation period was for 2 hours at 37° C in phosphate buffer at pH 8.0. A control experiment was included using 100 I.U. of PMS incubated with varying amounts of *p*-benzoquinone. The results obtained are shown in Table 4. 2:6-dimethylquinone (0.1 mg.)

TABLE 4. INACTIVATION OF PMS BY VARIOUS QUINONES

Compound	Dose per rat	No. of rats	Ovarian wt. (mg.)
PMS control	100 i.u.	4	135 (013–154)
+BQ	0.1 mg.	4	16 (14–18)
+BQ	0.05 mg.	4	20 (16–22)
+BQ	0.025 mg.	4	30 (24–33)
+PQ	0.1 mg.	2	22 (22, 22)
+PQ	0.05 mg.	2	25 (24, 25)
+PQ	0.025 mg.	2	78 (59, 96)
+MQ	0.1 mg.	3	33 (29–37)
+MQ	0.05 mg.	3	96 (78–106)
+MQ	0.025 mg.	3	150 (121–196)
+2:5 DMQ	0.1 mg.	2	98 (82, 114)
+2:5 DMQ	0.05 mg.	2	110 (100, 120)
+2:5 DMQ	0.025 mg.	2	96 (79, 112)
+TMQ	0.05 mg.	4	133 (113–150)

All incubations with PMS were made at pH 8.0 for 2 hours in air at 37° C.
BQ—p-benzoquinone.
PQ—phenylquinone.
MQ—methylquinone.
2:5 DMQ—2:5 dimethylbenzoquinone.
TMQ—tetramethylbenzoquinone.
Ovarian weights are the mean with the range or the individual weights in parentheses.

did not inactivate PMS (100 I.U.) when assayed in hooded rats under the same conditions of incubation.

The introduction of methyl groups into the quinone ring led to a marked reduction in activity. The monomethyl compound was approximately one-quarter as active as *p*-benzoquinone. 2:5- and 2:6-dimethylbenzoquinones were both without activity at a level of 0.1 mg. Phenylbenzoquinone was about twice as active as the monomethyl compound, and on a molar basis approached the activity of *p*-benzoquinone.

Reaction Products of p-Benzoquinone

In the experiments described above it was observed that during the incubation period both the hydroquinone and quinone solutions developed a distinct reddish-brown tint. Whereas this change occurred with both compounds in air or oxygen and became more pronounced as the alkalinity of the incubation media was increased, only the quinone solution became colored in a nitrogen atmosphere. The relationship of this color development to the antigonadotrophic action of the quinone solutions was therefore investigated.

In preliminary experiments U.V. absorption measurements showed that three successive extractions of a freshly prepared aqueous solution of *p*-benzoquinone (pH 6.0–8.0) with an equal volume of ether removed essentially all the quinone. The aqueous residue was completely colorless, and remained so on standing. However, when the colored solutions obtained by incubation (in air or nitrogen) were similarly extracted, the brown substances remained in the aqueous portion. This separation procedure was

used to determine the contributions of the ether-soluble and ether-insoluble fractions to the total inhibitory activity of the quinone solutions.

It was found that when a freshly prepared aqueous solution (pH 8.0) of *p*-benzoquinone was immediately extracted with ether, all the PMS inactivating substance was removed. However, when the quinone solution was incubated alone at 37° C prior to the extraction, the ether no longer removed all the antigonadotrophic activity from the aqueous portion. The ether-insoluble activity increased as the time of incubation was extended. After 3½ hours at 37° C at pH 8.0 the total activity of the "aged" solution was almost entirely retained in the aqueous portion, when extracted with ether. However, the total activity after this period of incubation was significantly less than that of freshly prepared solutions of *p*-benzoquinone. These experiments indicated that after 3½ hours incubation the *p*-benzoquinone had been converted to non-ether extractable substances, and that the latter were able to inactivate PMS, although not to the same extent as the parent quinone. Under the conditions of incubation, quinone readily formed the polymeric "humic acid" and the PMS inactivating properties of this material were therefore investigated.

Experiments with Quinone "Humic Acid"

Preparation of "humic acid"

A solution of *p*-benzoquinone (5 gm.) in diethyl ether (100 ml.) was shaken with 250 ml. of 0.1 M phosphate buffer at pH 7.0, and then refluxed (35° C) for 6½ hours. After standing overnight, the ether layer was separated and the dark brown aqueous portion was extracted four times with 100 ml. of ether. The final extracts were practically colorless. The aqueous solution was acidified to pH 1.0 with hydrochloric acid and allowed to stand overnight. The dark brown precipitate which formed was removed, washed well with 1 N hydrochloric acid and water, and dried *in vacuo* over KOH. A dark brown glassy solid (1.3 gm.) was obtained. It was insoluble in dilute acids but readily soluble in aqueous media at pH 8.0.

Biological assay

Known amounts of the "humic acid" were dissolved in phosphate buffer at pH 8.0 and incubated with PMS at 37 °C for 2 hours in air. Rats were given injections of these solutions in the manner previously described. The dose given and the ovarian and uterine weights of these animals at autopsy are shown in Table 5.

A comparison of the data in this table with that for *p*-benzoquinone in Table 3 indicates that the isolated "humic acid" was about one-tenth as active as freshly prepared *p*-benzoquinone solutions. The uteri of the animals receiving PMS with "humic acid" were large and filled with fluid, of the type noted previously in rats treated with PMS partially inactivated with *p*-benzoquinone.

DISCUSSION

The work reported here deals with the relationship of the products and reactions shown in Equation 1 to the inactivation of PMS gonadotrophin.

TABLE 5. INACTIVATION OF PMS BY QUINONE "HUMIC ACID"

	Dose per rat*	Ovarian wt. (mg.)	Uterine wt. (mg.)
PMS control	100 i.u.	148 (129, 167)	140 (131, 148)
+humic acid	0.5 mg.	21 (20, 21)	326 (269, 383)
+humic acid	0.25 mg.	24 (20, 28)	178 (129, 226)
+humic acid	0.1 mg.	37 (35, 39)	184 (173, 194)

All incubations with PMS were made at pH 8.0 for 2 hours in air at 37° C.
* 2 rats per group.
Ovarian and uterine weights are the mean with the individual weights in parentheses.

$$\text{Hydroquinone} \xrightarrow[(a)]{O_2} \text{Quinone} \xrightarrow[(b)]{} \text{"humic acid."} \qquad \text{Eq. 1}$$

The data in Figure 1 and Table 2 indicate that hydroquinone must first be oxidized to the quinone (Eq. 1(a)) before it can inactivate PMS gonadotrophin. The rate of this oxidation is markedly dependent on the pH and increases rapidly as the medium is made more alkaline. The inability of hydroquinone to inactivate PMS in air in the pH range 6.5–7.5 (in which range quinone is quite effective) can therefore be attributed to the slow rate at which it is oxidized to the quinone.

In addition the degree of inactivation of PMS with quinone, although not dependent on oxygen, also increases as the incubation medium is made more alkaline (Fig. 2 and Table 3). The results with the hydroquinones can therefore be explained as being due to the dual effects of the hydroxyl ion concentration, in the first place on the rate of oxidation to the quinone and secondly on the rate of reaction of the quinone so formed with the PMS.

Quinones may react with both sulfhydryl and amino groups by substitution in the quinone ring, and when there is competition between these groups for quinone the sulfhydryl group usually reacts first (3). A similar reaction may take place between quinone and PMS, either with sulfhydryl groups (if present), or with amino groups in the hormone.

In the present study the development of a brown color during the incubation of quinone-containing solutions indicated the formation of "humic acids" (Eq. 1(b)). This color change occurred more rapidly as the alkalinity of the medium increased, and was not dependent on the presence of oxygen. When acidified these brown solutions yielded a dark amorphous precipitate and an almost colorless supernatant. It was thought that these secondary reaction products might contribute to, or be mainly responsible for the inhibitory activity of an incubated quinone solution; however, although the quinone "humic acid" did reduce the biological activity of PMS, it was less effective than the parent quinone (Table 5). It is possible that "humic acid" as an anionic polymer forms an ionic complex with PMS in virtue of the basic groups which the latter contains, and in the process masks groups essential for the biological activity of the gonadotrophin. The relative degree of inactivation of PMS by quinone and its "humic acid" may be contrasted with the results of Roseman and Dorfman (12) who found that the

antihyaluronidase activity of the "humic acid" from *p*-benzoquinone was much greater than that of the parent quinone.

The present work supports and in part explains the original observations of Noble and Graham (2, 13, 14). It differs, however, in that under the incubation conditions described in this paper the dimethyl hydroquinones and quinones, gentisic acid and other compounds were, with a few exceptions, found to be inactive. This difference may be related to the purification of the compounds immediately before use, and to the use of buffered solutions to stabilize the pH.

Rosen and Millman (15, 16) have reported on the inactivation of PMS by a number of hydroquinones. In the present study many of these compounds were found to be inactive. This difference may be partially due to the much greater dose level used by these workers (0.6 mg./100 I.U. of PMS). Also the incubations were apparently done in air, and it seems probable that the activity reported was in fact due to the prior formation of the quinones, and possibly their "humic acids."

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A COMPARISON OF THE RATES AT WHICH THYROTROPHIN AND THE HUMAN ABNORMAL THYROID STIMULATOR DISAPPEAR FROM THE CIRCULATING BLOOD OF THE RAT

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ABSTRACT

Human serum, obtained from a thyrotoxic patient and containing a high concentration of the abnormal thyroid stimulator, was injected intravenously into rats. The amount of the abnormal thyroid stimulator remaining in the rats' blood after 1, 2 and 6 hours was compared with the amount present 2 minutes after injection. The half-life was 7.5 hours.

Similar experiments were conducted with U.S.P. Thyrotropin Reference Substance and human pituitary thyrotrophin obtained from a euthyroid subject. One hour after injection, the level of both these preparations had fallen to less than 5% of the 2-minute level.

It is suggested that the prolonged life of the abnormal thyroid stimulator in circulating blood is responsible for the prolonged nature of the response which it elicits.

IT HAS been shown that the blood of some thyrotoxic patients contains a thyroid-stimulating substance with a time course of action which differs from that of thyrotrophin (1, 2). The amount of this material present varies widely and in many thyrotoxic patients its presence can be demonstrated only after fractionation of the serum proteins (3). In occasional patients high concentrations are present. We have recently had two such patients and this has provided us with sufficient material to enable us to compare the rates at which thyrotrophin and the abnormal thyroid stimulator disappear from the circulating blood of the rat.

To determine whether or not there is a species difference between human and bovine thyrotrophin in this respect, human pituitary thyrotrophin was studied in addition to standard thyrotrophin (bovine) and the abnormal thyroid stimulator.

METHODS

Terminology

In this paper the term "thyrotrophin" is reserved for thyroid-stimulating hormone which elicits the "normal response," as previously defined (1). Such material is present in bovine, rat and euthyroid human pituitary glands and in the blood of hypothyroid people and rats (1).

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The material eliciting the "abnormal response" (1), is called the "abnormal thyroid stimulator." A more descriptive name for this material would be "long-acting thyroid stimulator," but the use of this name is being deferred meanwhile as future work may suggest an even better name. The abnormal thyroid stimulator, so far, has been found only in the blood of patients with Graves' Disease.

The term "thyroid-stimulating hormone" or TSH is used for both thyrotrophin and the abnormal thyroid stimulator, but this usage does not imply that the latter necessarily acts directly on the thyroid.

Injection and bleeding of rats

Male rats weighing about 140 gm. were used, all having received 30 μg. of L-thyroxine daily for the preceding two days to inhibit their own thyrotrophin secretion.

The abnormal thyroid stimulator (serum from thyrotoxic patient W.R.P.) was injected via a tail vessel, the volume being 2.0 ml. per rat. Immediately before the injection the rats were bled 1.0 ml. from a cut tail vessel to reduce their blood volume and so to minimize the increase caused by the injection. The bleeding and injection were performed under light ether anaesthesia, with the rat warmed by an overhead lamp to dilate the tail vessels.

The standard thyrotrophin (U.S.P. Thyrotropin Reference Substance) and the human pituitary thyrotrophin (saline extract of whole pituitary from autopsy of a case of status asthmaticus) were injected in exactly the same manner as the abnormal thyroid stimulator, each injection consisting of the chosen amount of thyrotrophin dissolved in 2.0 ml. of euthyroid human serum. The amount of standard thyrotrophin injected into each rat was 0.4 I.U. The amount of human thyrotrophin per injection is not known exactly, but a rough assay indicated that it was of the order of 0.07 I.U.

In every instance, three rats were used to provide the blood for each plasma "pool" to be assayed. Separate animals were used for each pool, no rat being bled more than once. The blood for the pools was collected from the rats 2 minutes, 1 hour, 2 hours or 6 hours after the injection of TSH. According to the time of bleeding the pools were designated "2-minute pool," "1-hour pool," etc.

In the studies of standard and human pituitary thyrotrophin 2-minute and 1-hour pools were collected. In the study of the abnormal thyroid stimulator pools were collected at 2 minutes, 1 hour, 2 hours and 6 hours.

For all bleedings the rats were under light ether anaesthesia. For 2-minute pools the rats were bled by heart puncture, this taking ½ to 1 minute to provide 4 to 5.5 ml. of blood. To prevent clotting, 0.2 ml. of heparin solution was injected into the heart before blood was withdrawn from the heart puncture. For 1 hour and later pools the rats were fully exsanguinated, the heart puncture being supplemented by thoractocomy, the procedure taking 1 to 2 minutes.

TSH assay method

The TSH assays were done by a method which is a slight modification of McKenzie's adaptation to mice (4) of the guinea pig plasma I^{131} measurements method (5). Mice give more variable responses than do guinea pigs. To offset this, when using mice we use larger numbers of animals and to avoid prohibitively increasing the time required for counting the plasma I^{131} samples, we use the mice in pairs, each 0.1 ml. mouse plasma I^{131} sample counted consisting of 0.05 ml. of blood from each of two mice. Thus the number of mice used is double the number of responses measured.

In the assays of plasma from rats receiving standard thyrotrophin and human pituitary thyrotrophin, 2-hour responses were measured in the assay mice, whereas 16-hour responses were measured in the assays of plasma from rats receiving the abnormal thy-

TABLE 1. ASSAYS OF THE TSH CONTENT OF RAT PLASMA 2 MINUTES AND 1 HOUR AFTER INTRAVENOUS INJECTION OF (A) STANDARD THYROTROPHIN AND (B) THE ABNORMAL THYROID STIMULATOR

Material injected into rats	Time from TSH injection to bleeding	TSH assay of pooled rat plasma				
		Dilution	N[1]	Mean response[2]	P for difference[3]	Estimated TSH content
(a) Standard Thyrotrophin, 0.4 I.U. in 2.0 ml. euthyroid serum	2 minutes	1 in 40	10	220	<0.001	100
	1 hour	undiluted	10	76		<2.5
(b) Abnormal thyroid stimulator, 2.0 ml. of W.R.P.'s serum	2 minutes	1 in 2	14	52	<0.05	100
	1 hour	undiluted	12	116		>50

[1] N = number of responses measured. Number of mice used = 2N.
[2] The response is the rise in the plasma I^{131} level of the mouse, expressed as a percentage of the initial value.
[3] Students' t test applied to the difference between the responses given by the 2-minute and the 1-hour rat plasma pools.

roid stimulator. In addition, some 2-hour responses were measured for the 1-hour rat plasma pool containing the abnormal thyroid stimulator, to enable determination of the response ratio (2-hour response divided by 16-hour response) (1). Similarly, some 16-hour responses were measured to enable determination of the response ratio of the 1-hour rat plasma pool containing standard thyrotrophin. Control injections of saline were made in all assays where 16-hour responses were measured, the response being the difference between the mean value elicited by the rat plasma pool and the mean value elicited by the saline (1).

TSH assay of the rat plasma pools

In the first experiment (Table 1), the 2-minute rat plasma pool containing standard thyrotrophin was diluted 1 in 40 prior to assay. Then the diluted 2-minute pool was compared with the undiluted 1-hour pool in several assays until the difference between the mean responses to the two pools was highly significant. The 2-minute and 1-hour rat plasma pools containing the abnormal thyroid stimulator were compared similarly but the 2-minute pool was diluted only 1 in 2.

In the second experiment (Table 2), which involved human pituitary thyrotrophin, a shortage of this material necessitated the adoption of a different procedure. The 2-minute pool was diluted 1 in 10 and assayed against two doses of standard thyrotrophin, the doses chosen giving responses which bracketted the response to the diluted pool. The potency of the pool was calculated in two ways, one assuming a linear log-dose-response

TABLE 2. ASSAYS OF THE TSH CONTENT OF RAT PLASMA 2 MINUTES AND 1 HOUR AFTER INTRAVENOUS INJECTION OF HUMAN EUTHYROID PITUITARY THYROTROPHIN

Material injected into rats	Time from TSH injection to bleeding	TSH assay of pooled rat plasma					
		Doses of standard	Responses to standard	Dilution of pool	Response to 0.3 ml. pool	Estimated TSH content	
						mU/ml.	%
Human pituitary	2 minutes	0.45 mU 0.15 mU	192 56	1 in 10	128	9.7 ±1.7[1]	100
13.5 mg. (≃0.07 I.U.) in 2.0 ml. of euthyroid serum	1 hour	0.15 mU	93	undiluted	22	<0.43 P <0.02[2]	<4.4

[1] Standard error.
[2] The probability that the TSH content was as high as 0.43 mU/ml.

TABLE 3. ASSAYS OF THE TSH CONTENT OF RAT PLASMA 2 MINUTES AND 2 HOURS AND 2 MINUTES AND 6 HOURS AFTER INTRAVENOUS INJECTION OF THE ABNORMAL THYROID STIMULATOR

Material injected into rats	Time from TSH injection to bleeding	TSH assay of pooled rat plasma			
		N	Mean response	Units of abnormal thyroid stimulator/0.3 ml.[1]	%
Abnormal thyroid stimulator, 2.0 ml. of W.R.P.'s serum	2 minutes	12	216	106	100
	2 hours	12	157	82	77
	2 minutes	8	486	195	100
	6 hours	8	237	113	58

[1] Unit of abnormal thyroid stimulator is an amount equal to that contained in 0.001 ml. of M.McC. III serum.

relationship, the other assuming a linear dose-response relationship. This is because the mouse dose-response relationship for thyrotrophin (6), like that of the guinea pig (5), is intermediate between log-dose-response linearity and dose-response-linearity over the range used. The curvature is slight over three-fold dosage ranges so the two potency estimates and their errors differ only slightly. In this instance the log-dose estimate was 9.0 mU/ml. $\pm$1.8 mU/ml. and the arithmetic-dose estimate was 10.3 mU/ml. $\pm$1.5 mU/ml., the value given in Table 2 being the mean of these. The 1-hour pool was assayed undiluted against a dosage of 0.13 mU of standard thyrotrophin.

In the third and fourth experiments (Table 3), 2-minute pools containing the abnormal thyroid stimulator were compared with 2-hour and 6-hour pools. The mean responses elicited were converted to "units" of abnormal thyroid stimulator by reading off the dose-response line for the abnormal thyroid stimulator (6). One unit of the abnormal thyroid stimulator is the amount contained in 0.001 ml. of M. McC. III serum.

Estimate of the half-life of the abnormal thyroid stimulator

This was done in two ways, both assuming exponential fall. Firstly, by making use of the 6-hour value and its fiducial limits (calculated by the method described in the appendix of reference 1). Secondly, by calculating the regression coefficient of the 2-minute, 1-hour, 2-hour and 6-hour values, assuming homogeneous variance.

RESULTS

Rate of disappearance of standard thyrotrophin

Table 1(a) shows the mean responses elicited by rat plasma collected 2 minutes and 1 hour after intravenous injection of standard thyrotrophin. This material (U.S.P. Thyrotropin Reference Substance) is prepared from bovine pituitary glands. Prior to assay, the 2-minute plasma was diluted 1 in 40, whereas the 1-hour plasma was assayed undiluted. The fortyfold dilution of the 2-minute plasma elicited a response significantly greater ($P < 0.001$) than that elicited by the undiluted 1-hour plasma. It is apparent that the level of the injected standard thyrotrophin in the rats' plasma at 1-hour was less than 2.5% of that at 2 minutes. This result is in agreement with the previous work of D'Angelo (7) and Levy and Solomon (8). The response ratio (1) of the 1-hour rat plasma was 2.9.

Rate of disappearance of human pituitary thyrotrophin

Table 2 shows the measurements of the thyrotrophin content of rat plasma collected 2 minutes and 1 hour after intravenous injection of human pituitary thyrotrophin. The amount present at 1 hour was less than 4.4% of the amount present at 2 minutes.

(c) *Rate of disappearance of the abnormal thyroid stimulator*

Table 1(b) shows the mean responses elicited by rat plasma collected 2 minutes and 1 hour after intravenous injection of 2.0 ml. of serum from thyrotoxic patient W.R.P. This serum elicited a 16-hour response of 1030, with a response ratio (1) of 0.43, on direct assay. The 2-minute rat plasma was diluted 1 in 2 before assay, the 1-hour rat plasma being assayed undiluted. The response to the 1-hour plasma was significantly greater ($P < 0.05$) than that to the twofold dilution of the 2-minute plasma. Both responses differed significantly ($P < 0.01$) from the saline control values.

The data indicate that the level of the injected abnormal thyroid stimulator in the rats' plasma at 1 hour was greater than 50% of that at 2 minutes. An approximate estimate of the actual value, obtained by making reference to the dose-response line for the abnormal thyroid stimulator in mice, was 96%. The response ratio of the 1-hour rat plasma was 0.31, showing that passage through the rat did not alter the time course of the response elicited by the abnormal thyroid stimulator when injected into mice.

Table 3 shows the mean responses elicited by rat plasma collected 2 minutes and 2 hours and 2 minutes and 6 hours after intravenous injection of 2.0 ml. of W.R.P.'s serum. The mean responses have been converted into units of abnormal thyroid stimulator (see "Methods. TSH assay of rat plasma pools"). It is seen that the concentration of abnormal thyroid stimulator in the rats' plasma had fallen to 77% of the 2-minute value by 2 hours and to 58% by 6 hours.

Figure 1 shows the amounts of the abnormal thyroid stimulator in the rat plasma at 1 hour, 2 hours and 6 hours, expressed as percentages of the 2-minute value. Assuming exponential fall, the 6-hour value indicates a half-life of 7.6 hours with fiducial limits of 22.2 hours and 3.72 hours for $P = 0.05$. The regression coefficient, calculated from the 2-minute, 1-hour, 2-hour and 6-hour values, assuming homogeneous variance, indicates a half-life of 7.4 hours with fiducial limits of 23.3 hours and 4.4 hours for $P = 0.05$. Figure 1 also shows the upper limits of the estimates of the levels of human pituitary thyrotrophin and standard thyrotrophin in the rat plasma at 1 hour, expressed as percentages of the 2-minute levels.

DISCUSSION

It is apparent that the life of the abnormal thyroid stimulator in circulating rat blood is much longer than that of either standard thyrotrophin or

human pituitary thyrotrophin. The prolonged life of the abnormal thyroid stimulator cannot be attributed to a species difference between human and bovine TSH.

The abnormal response has been described as a "delayed" response (9, 2). While it is true that the time at which the response reaches its maximum value is delayed, the time of onset of the response is not delayed (see Fig. 1, reference 1), therefore it is better described as a "prolonged" response.

It seems likely that the prolonged nature of the abnormal response is due to the prolonged life of the abnormal thyroid stimulator in circulating blood.

We have not been able to measure the life in circulating rat blood of

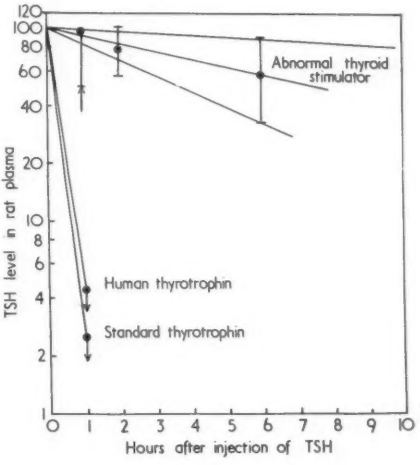

FIG. 1. The TSH content of rat plasma at various times after intravenous injection of human pituitary thyrotrophin, standard thyrotrophin and the abnormal thyroid stimulator (patient, W.R.P.'s serum). The limits of error shown are for $P = 0.05$. The arrows indicate that the associated values are significantly greater or less than the points shown (see Table 1).

human serum thyrotrophin from non-thyrotoxic subjects, as we have no such sera of sufficient potency. Therefore, it has not been possible to prove that the thyrotrophin present in the blood of patients with myxedema and cretinism does not also have a prolonged stay in blood. It seems unlikely, however, that human pituitary thyrotrophin and human myxedema serum thyrotrophin could differ much in their time of stay in blood when they both have the same short time course of action in the TSH assay, in contrast to the abnormal thyroid stimulator which has a prolonged stay in blood and a prolonged time course of action in the assay.

The dose-response relationship of the abnormal thyroid stimulator (6) is similar to that which would be predicted for normal thyrotrophin having a prolonged life in the blood. If the abnormal thyroid stimulator acts directly on the thyroid (which is not yet established), then there is no evidence at present that its mechanism of action differs from that of normal thyrotrophin.

The site of production of the abnormal thyroid stimulator remains unknown.

Acknowledgments

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CHANGES IN THE METABOLISM OF S^{35}-LABELED SULFATE ASSOCIATED WITH RENAL CALCIFICATION

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ABSTRACT

Renal calcification was produced in 50 laboratory mice of mixed sexes by the administration of parathyroid extract or 10% calcium gluconate. 10 μc. of $Na_2S^{35}O_4$ was injected into each mouse 24 hours prior to sacrifice. The renal calcification resulting from the administration of PTE was accompanied by an increase in the amount of bound S^{35}. This increase was proportionate to the degree of calcification. No change in sulfate binding accompanied the calcification produced by calcium gluconate.

THE calcification produced by the administration of parathyroid extract (PTE) is thought to be a direct result of the hypercalcemia produced by PTE (1). However, current studies in our laboratory have demonstrated a basic histologic difference between the calcification produced by PTE and that produced by 10% calcium gluconate. This observed difference is not consistent with the concept that calcification resulting from administration of PTE is simply a result of elevated serum calcium levels, and was considered worthy of further investigation.

It has been stated that PTE has a direct effect on the connective tissue ground substance of the rat kidney, and that this change precedes the deposition of calcium in these areas (2). With this thought in mind, we have studied the uptake of S^{35}-labeled sulfate by the mouse kidney in an attempt to differentiate the calcification produced by PTE from that produced by calcium gluconate. This is based on the assumption that the majority of S^{35}-labeled sulfate fixed by tissue is deposited as ester sulfate in chondroitin sulfuric acid (CSA), with little activity being found in other sulfated compounds (3). Since CSA is a major component of the connective tissue ground substance, any change in the uptake of administered S^{35} would imply a change in the metabolism of the connective tissue ground substance.

MATERIALS AND METHODS

Fifty laboratory mice of mixed sexes, each weighing approximately 20 gm., were used for these experiments. They were divided at random into 3 groups: 1) 20 mice; 2) 10

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mice; and 3) 20 mice, 12 of which died during the experiment. The mice were then injected intraperitoneally over the 5 day experimental period according to one of the following regimes: 1) 200 units of PTE (Eli Lilly Co.), the total amount being given in gradually increasing doses over the experimental period; 2) a control solution consisting of 0.2 gm.% phenol and 0.6 gm.% bovine albumin in normal saline, which corresponded to the concentration of phenol and protein in the PTE, and was injected in volumes equal to the PTE; and 3) 10% calcium gluconate, 0.4 cc./day for 5 days, or a total of 2.0 cc.

On the fifth experimental day, in addition to the experimental injection, the mice were injected subcutaneously with 10 μc. of $Na_2S^{35}O_4$. They were sacrificed 24 hours later by severing the cervical spinal cord. One kidney was removed, fixed in absolute alcohol, and subsequently stained for calcium phosphate and/or calcium carbonate by the von Kossa method. Three slices taken from the remaining kidney were weighed and placed in separate counting vials. The kidney slices were dissolved in hyamine, and the activity determined in a Packard Tri-Carb Liquid Scintillation Spectrometer (4). The S^{35} activity was expressed as counts/mg. wet weight/min. The mean of three determinations from each kidney was used as the final value.

The amount of tissue calcium was expressed semi-quantitatively as determined from the sections stained with the von Kossa method. Four values were used; 0, 1 plus, 2 plus, and 3 plus. These values referred respectively to the complete absence of calcium, minimal calcification, moderate calcification, and extensive calcification. The slides were evaluated independently by 3 observers.

RESULTS

Injection of the control solution appeared to be innocuous, and there were no deaths in this group of animals. The mortality among the animals receiving PTE was minimal, but in contrast to the control group, many of these animals seemed to be ill when sacrificed. There was a remarkable correlation in this group between the morbidity and the degree of renal calcification observed microscopically. The mortality among the animals receiving calcium gluconate was quite high.

The experimental results are illustrated in Figure 1. The amount of S^{35}-labeled sulfate bound is charted against the degree of renal calcification for each animal. Renal calcification did not develop in the control animals, and the amount of S^{35}-labeled sulfate fixed by the kidney was quite constant within this group. On the contrary, approximately two-thirds of the mice receiving PTE exhibited varying amounts of calcific deposits; the more extensive the calcification, the more S^{35}-labeled sulfate was fixed by the kidney. There were six animals receiving PTE that did not demonstrate calcification, and all had levels of bound S^{35} comparable to those of the control group. Finally, renal calcification was observed in every animal receiving calcium gluconate. However, in contrast to the calcification produced by PTE, there seemed to be no correlation between radioactive sulfate uptake and the degree of calcification. The amount of S^{35}-labeled sulfate bound by the kidney was quite comparable to that observed in the control group.

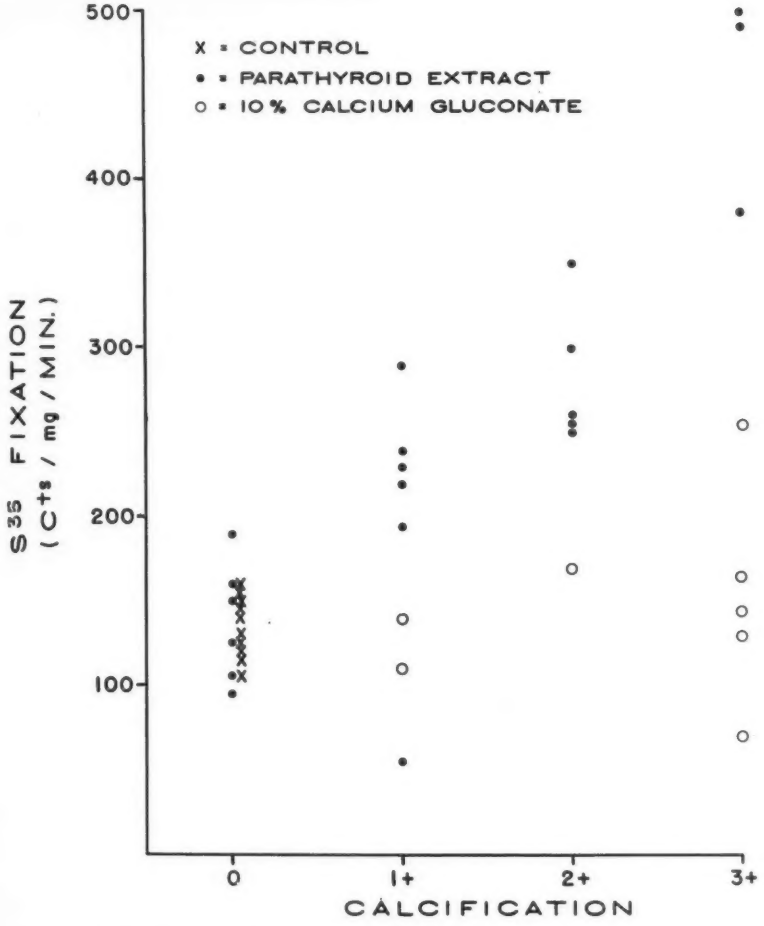

FIG. 1. Relationship between sulfate fixation and calcification charted for each experimental animal.

DISCUSSION

Renal calcification produced by PTE is associated with an increase in sulfate binding; the more extensive the calcification, the greater the degree of sulfate binding. Renal calcification produced by calcium gluconate was not accompanied by an increase in sulfate binding. Parathyroid extract had no effect on sulfate metabolism when calcification did not also occur. Consequently, it is possible that the observed changes in sulfate metabolism produced by PTE were secondary to the deposition of calcium in the soft tissue. However, since changes in sulfate metabolism did not occur

when the renal calcification was produced by calcium gluconate, it may not be a nonspecific response, and perhaps is a change in the state of the connective tissue which accompanies PTE-induced nephrocalcinosis.

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PREPARATION AND METABOLISM OF IODINATED PEPTIDES[1]

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ABSTRACT

A method of preparation and of I^{131}-labeling of L-leucyl-L-mono- or diiodotyrosine, and glycyl-L-mono- or diiodotyrosine is described.

Studies with sheep thyroid and liver have been performed. A peptidase which hydrolyzes the iodinated dipeptides is present in liver and thyroid microsomes and in the cell sap of these preparations. A tissue deiodinase was also demonstrated, but it could not be shown that this was active prior to hydrolysis of the dipeptide.

ABNORMAL iodinated substances have been reported in the blood of patients with carcinoma (1, 2), congenital goiter (3), and chronic thyroiditis (4). In this laboratory a metabolically inactive protein or polypeptide has been described in the blood of certain patients with congenital goiter and hypothyroidism. This abnormal complex contains peptide-linked iodotyrosines and iodothyronines, moves electrophoretically with albumin, is poorly extracted from acidified serum into *n*-butanol, and is almost immobile in various chromatographic systems using butanol.

A similar serum iodine component has been found in approximately 60% of patients with functional carcinoma of the thyroid (2). This substance, designated Compound X by Robbins who first described it, has an electrophoretic mobility similar to albumin. When incubated with trypsin or alkali it yields monoiodotyrosine and a small amount of thyroxine. It is also poorly soluble in butanol. A significant amount of iodoprotein which is insoluble in acid butanol may also appear in the blood in the course of chronic thyroiditis.

These observations have stimulated a study of the metabolism of iodinated peptides. This report concerns the preparation and degradation of the iodinated peptides, L-leucyl-L-mono- or diiodotyrosine, and glycyl-L-mono- or diiodotyrosine.

METHODS

Preparation of iodinated L-leucyl-L-tyrosine

L-Leucyl-L-tyrosine[3] was iodinated by methods similar to those described previously for the iodination of tyrosine (5). Iodine prepared in an ether phase was incubated with

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leucyl-tyrosine in alkaline solution (1.6 mg./ml.). When the iodine was prepared with concentrated peroxide a better yield of the iodopeptide was obtained. The reason for this is unexplained. When the molar ratio of iodine to LT[4] is 6/1, one obtains mostly Leu-MIT. If the ratio of iodine is slightly increased, a mixture of Leu-MIT and Leu-DIT is formed. With increasingly higher concentrations of iodine, more Leu-DIT is produced. After incubation for 12–15 hours, the solution is dried by an air stream. The iodinated products are then dissolved in concentrated ethanol-ammonia and purified by chromatography. The elution is carried out with 0.9% saline at a slightly alkaline pH.

The addition of I^{131} to the sodium iodide during the first step permits formation of labeled iodinated L-leucyl-L-tyrosine. The yield of labeled iodinated leucyl-tyrosine is poor. One must use 5–7 mc. of I^{131} in order to obtain microcurie amounts of Leu-MIT or Leu-DIT. These labeled peptides do not deteriorate when kept frozen.

Preparation of iodinated glycyl-L-tyrosine

The same procedure as for LT has been followed for the iodination of glycyl-L-tyrosine.[3] Glycyl-tyrosine prepared in an alkaline solution (1.24 mg./ml.) was incubated with iodine. With a molar ratio of iodine to GT of 6/1, G-MIT was chiefly formed. Variations in the ratio gave a mixture of G-MIT and G-DIT, or G-DIT.

Solvents

Several solvents have been used to characterize the iodinated peptide in one or two-dimensional chromatography:

1) n-butyl alcohol-acetic acid-water (250:60:250 v/v),
2) collidine-water (35:100 v/v) in atmosphere of ammonia,
3) pyridine-butanol-acetic acid-water (20:36:6:24 v/v),
4) phenol-water (100:20 v/v, Mallinckrodt liquid phenol), in the presence of HCN,
5) 2,6-lutidine-collidine-water (1:1:1) with 1–2% diethylamine.

In the butanol-acetic acid solvent system, both Leu-MIT and Leu-DIT occupy positions overlapping the thyroxine zone, but they were distinct from thyroxine in several other solvent systems. No evidence was found for the formation of thyroxine under the condition of iodination, as determined by two-dimensional chromatography. Accordingly, butanol-acetic acid descending chromatography was employed for routine studies. The leucyl-tyrosine contained traces of leucine and tyrosine. The presence of the latter explains the formation of some MIT and DIT, as shown in Figure 1.

No solvent system was found which clearly separated Leu-MIT from Leu-DIT. Proof of the presence of each was sought by hydrolysis and chromatography of eluates from various segments of preparative chromatograms in the zone corresponding to iodinated LT. Crude pancreatin (5 mg.) suspended in bicarbonate buffer at pH 8.3 was used to hydrolyze the eluates. Following hydrolysis of the labeled iodinated LT, labeled MIT, DIT, or a mixture of MIT and DIT was obtained (Fig. 2).

If the eluate was from the proximal segment of the I^{131}-LT zone, hydrolysis yielded primarily labeled MIT, and if from the distal zone, labeled DIT was obtained (Fig. 3).

Iodinated glycyl-tyrosine overlaps MIT in one-dimensional chromatography in the usual systems. Descending chromatography, using pyridine-butanol-acetic acid system, gives a satisfactory separation from MIT. However, in these conditions, the iodide spot overlaps MIT and DIT. For this reason, separation and routine studies of this

[4] Abbreviations: LT: L-leucyl-L-tyrosine; I^{131}-LT: I^{131}-labeled LT; Leu-MIT or Leu-DIT: leucyl-L-mono- or diiodotyrosine; GT: glycyl-L-tyrosine; G-MIT or G-DIT: glycyl-L-mono- or diiodotyrosine; MIT: monoiodotyrosine; DIT: diiodotyrosine; I^-: iodide; BA: n-butanol-glacial acetic acid-water solvent for chromatography; Tris: tris hydroxymethyl aminomethane.

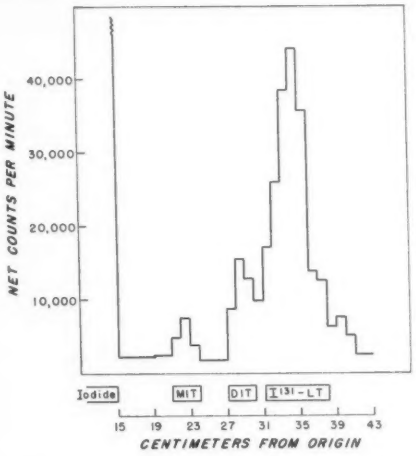

FIG. 1. Radiochromatography of I^{131}-LT in butanol-acetic acid solvent system.

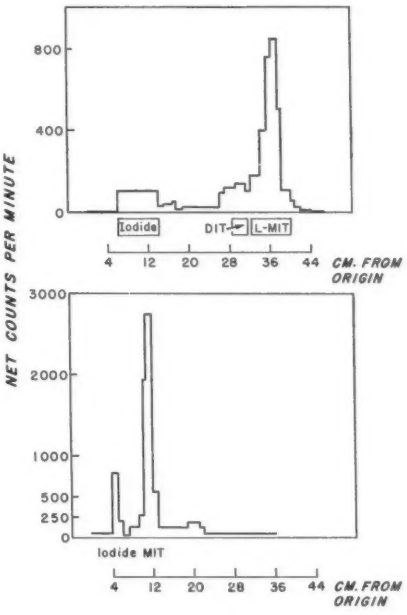

FIG. 2. Action of crude pancreatin on an eluate of I^{131}-LT (butanol-acetic acid solvent). Above: Before hydrolysis. Below: After hydrolysis.

peptide have been carried out in two-dimensional chromatography, using the butanol-acetic acid system in the first direction, and pyridine-butanol-acetic acid-water in the second.

Tissue Studies

Sheep liver and thyroids collected at the time of slaughter were brought at once on ice to the laboratory. The tissues were dissected free from neighboring structures, prepared and fractionated as previously described (6). Homogenization was carried out in all glass Potter-Elvehjem homogenizer tubes on ice in 0.38M sucrose solution buffered to pH 7.3 with 0.1N Tris and containing 0.005M $MgCl_2$, 0.025M KCl ,and 0.28M $KHCO_3$. Thyroid tissues were treated first for a few seconds in the Servall Omnimixer. Initially the homogenates contained 300 mg. whole tissue per ml. Tissue particulate fractions were prepared by centrifuging at 12,000×g for 10 minutes at 0° C. The supernatant collected from this first centrifugation was then spun at 105,000×g for 1 hour in the Spinco Model "L" centrifuge. The term "soluble fraction" was applied to the supernatant solution from this last centrifugation. The microsomal pellet was washed with buffered cold sucrose and usually resuspended in the 0.38M sucrose medium. Separation between the "soluble fraction" and the microsomal pellet was carried out as carefully as possible. The incubation mixture usually contained 0.5–1 ml. of tissue particulate fraction, 10^{-3}M thiouracil, I^{131}-leucyl-tyrosine (100,000–120,000 cpm in a well-type scintillation counter equipped with a pulse-height analyzer) or I^{131}-glycyl-tyrosine (20,000–40,000 cpm), and Tris buffer at pH 7.4 for a total volume in the flask of 4 ml. The

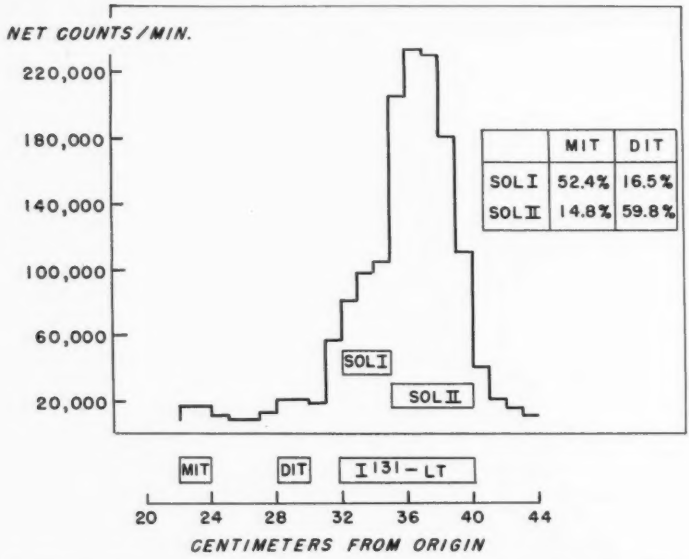

FIG. 3. Chromatography in butanol-acetic acid of an eluate of I^{131}-LT. Study of the partition of Leu-MIT and Leu-DIT in the I^{131}-LT zone. The indicated zones were eluted from the chromatogram and rechromatographed to give the results shown in the insert.

incubation time was 30 minutes. pH values in the flasks were frequently measured. The reaction was stopped by adding an excess of MIT and DIT, and heating at 100° C for 1 minute.

An aliquot from each flask (2,000–10,000 cpm) was applied linearly on Whatman No. 3 paper strips 3 cm. wide for descending chromatography in the studies with iodinated LT, and for two-dimensional chromatography with iodinated GT. In the latter case, butanol extraction of the total volume of each flask was performed. Phenolic compounds were stained with diazotized sulfanilic acid, and the iodide with palladium chloride. The zones of interest were measured in a scintillation counter and the results expressed in per cent of the total labeled iodine of the strip. Approximately 10% of the labeled iodine was found elsewhere than in the iodide, MIT, DIT, and labeled LT zones.

RESULTS

In these studies two enzymatic systems have to be considered. The first has peptidase activity which splits the iodinated dipeptide directly; the second is a deiodinating system which acts upon MIT and DIT, and possibly also directly on the iodinated peptide.

Properties of peptidase system

Both microsomes and soluble fractions of thyroid and liver homogenates show peptidase activity toward the iodinated L-leucyl-L-tyrosine (Table 1). Heating for one minute at 100° C completely abolished the activity of the enzyme. Peptidase activity was not abolished by versene at 5×10^{-3}M.

TABLE 1. ENZYME ACTIVITY OF TISSUE PARTICULATE FRACTIONS

Hydrolysis of Leu-MIT. Activity expressed in per cent of the total labeled iodine of the chromatogram.

	I^-	MIT	DIT	Leu-MIT
Thyroid				
Microsomes	12.2	61.6	7.0	3.7
Soluble Fraction	33.9	54.1	2.0	1.2
Microsomes + Soluble Fraction	72.0	10.4	4.6	1.9
Control	10.5	2.2	1.5	74.3
Liver				
Microsomes	9.7	71.7	6.7	2.4
Soluble Fraction	11.0	77.3	1.3	1.7
Microsomes + Soluble Fraction	17.3	62.2	4.1	3.9
Control	10.0	3.9	1.2	71.4

The addition of a variety of metal cations, such as Mn^{++}, Mg^{++}, Hg^{++}, Co^{++}, Ca^{++}, Cd^{++}, Cu^{++}, and Zn^{++} in their chloride form was studied. Hydrolysis of the peptides by the microsomes was inhibited by 10^{-3}M Hg^{++}, but hydrolysis by whole homogenate or soluble fraction was little inhibited by Hg^{++}, presumably because of preferential binding of the Hg^{++} by groups other than the hydrolyzing enzyme (Table 2). Cadmium ion also inhibited hydrolysis by microsomes.

The peptidase activity of thyroid microsomes diminished with decreasing concentrations of tissue (Fig. 4). Addition of an excess of LT as substrate, even in high concentrations (50–1500 μg. of LT per flask) failed to inhibit the splitting of I^{131}-LT (Fig. 4). Human serum had a high peptidase activity; the rate of hydrolysis was at least as great as in the studies with liver and thyroid homogenates.

Typical results with thyroid and liver microsomes or soluble fractions on iodinated glycyl-tyrosine are shown in Table 3. They are quite similar to those described for I^{131}-LT.

TABLE 2. EFFECT OF Hg^{++} ON HYDROLYSIS AND DEIODINATION OF IODINATED LEUCYL-TYROSINE

Activity expressed in per cent of the total labeled iodine of the chromatogram developed in a butanol-acetic acid-solvent system. Hg^{++} 10^{-3} M. In Experiment B the soluble fraction was diluted 1 to 5.

	I^-	MIT	DIT	I^{131}-LT
A. Microsomes	2.5	52.7	16.4	25.6
Microsomes + Hg^{++}	2.1	1.3	4.0	90.0
Control	2.3	1.2	1.7	92.9
B. Soluble fraction	10.3	66.4	13.2	2.9
Soluble fraction + Hg^{++}	10.8	52.4	12.8	15.6
Control	12.0	14.4	2.3	62.4
C. 8,000 × g thyroid supernatant	29.3	54.7	10.5	1.7
8,000 × g thyroid supernatant + Hg^{++}	7.7	76.1	11.8	2.7
Control	8.2	1.6	1.7	84.2

TABLE 3. HYDROLYSIS AND DEIODINATION OF IODINATED GLYCYL-TYROSINE BY TISSUE FRACTIONS

Activity expressed in per cent of the total labeled iodine of the chromatogram.

	I^-	MIT	DIT	G-MIT
Thyroid				
Microsomes	8.8	84.6	2.6	4.1
Microsomes + Soluble Fraction	34.0	61.3	1.7	3.0
Microsomes + Hg^{++}	3.4	45.8	3.2	47.6
Microsomes + Soluble Fraction + Hg^{++}	3.3	45.0	2.4	49.2
Control	2.0	3.2	1.0	93.8
Liver				
Microsomes	3.4	93.6	1.5	4.1
Microsomes + Soluble Fraction	15.6	81.7	2.3	0.4
Microsomes + Hg^{++}	1.8	48.2	0.6	49.4
Microsomes + Soluble Fraction + Hg^{++}	1.7	40.5	1.4	56.3
Control	0.1	2.2	0.3	96.4

Properties of deiodinating system

As shown in Tables 1, 2, and 3, thyroid and liver microsomes did not deiodinate iodinated peptides. There was a moderate amount of deiodination by the soluble fraction alone. Deiodination proceeded rapidly when using microsomes and soluble fraction from thyroid together. There was moderate deiodination with a similar preparation of liver. Deiodination was inhibited by 10^{-4}M Hg^{++} and Cd^{++}. No effect of other metals or versene was observed. Heating for 1 minute stopped the reaction.

COMMENTS

The feasibility of preparing iodinated derivatives of leucyl-tyrosine and glycyl-tyrosine has been demonstrated by these studies.

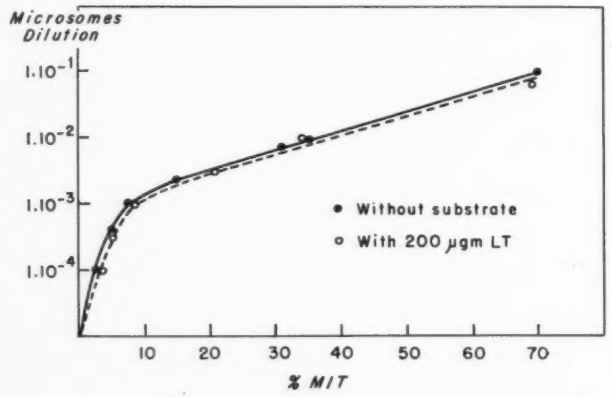

FIG. 4. Hydrolysis of Leu-MIT as affected by varying amounts of enzyme. Abscissa: Per cent of substrate I^{131}-labeled Leu-MIT hydrolyzed to MIT. Ordinate: Fraction of the pellet of microsomes diluted with 7 ml. of 0.38M sucrose.

Iodination of both dipeptides is easily accomplished under the conditions of preparation described above, but the yield of the reaction is poor, particularly for I^{131}-labeled GT. The length of time of incubation of the dipeptide with the iodine does not affect significantly the labeling of the LT and GT, but this did increase the formation of MIT and DIT from the small amounts of free tyrosine contaminating the dipeptides. The iodinated peptides are stable in the cold and to short periods of boiling. When purified they are chromatographically homogeneous.

Peptidase activity which splits iodinated LT and GT is found both in the microsomes and in the soluble fraction of liver and thyroid. When the incubating system contains both microsomes and soluble fraction, MIT (or DIT) which is formed is rapidly deiodinated. This explains the lower value of MIT (or DIT), compared with those obtained with the microsomes of the soluble fraction alone. The peptidase system is thermolabile. Versene is ineffective in blocking the reaction. As shown in Tables 2 and 3, Hg strongly inhibits the hydrolysis of the I^{131}-LT and GT by the microsomes. An excess of each uniodinated dipeptide failed to inhibit hydrolysis of the iodinated compound, even at high concentrations.

An inhibitor of the peptidase system, not acting on the deiodinase, has been sought unsuccessfully. For this reason, it has not been ascertained whether the deiodinating system is acting prior to hydrolysis.

Laver and Trikojus (7) found an intracellular peptidase in hog thyroids, located both in the microsomes and in the microsomal supernatant. Hydrolysis of uniodinated peptides, as leucyl-tyrosine and glycyl-tyrosine, by purified thyroid protease were unsuccessful (8).

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THE PHYSIOLOGICAL DISPOSITION IN THE RAT OF THE ACETIC AND PROPIONIC ACID ANALOGUES OF THYROXINE AND TRIIODOTHYRONINE[1]

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ABSTRACT

The distribution and excretion in the rat of tetraiodothyroacetic acid and tetraiodothyropropionic acid were compared with that of L-thyroxine; in addition, the distribution and excretion of 3,5,3′-triiodothyroacetic acid and 3,5,3′-triiodothyropropionic acid were compared with that of 3,5,3′L-triiodothyronine. Thyroxine and triiodothyronine disappeared from the blood more rapidly and were concentrated in the peripheral tissues to a greater extent than were their respective acetic- and propionic-acid analogues. The ratio of concentration in tissue to the concentration in plasma was lower with these analogues than with the parent compounds in all tissues examined and at all time intervals studied. The differences observed were of such magnitude as to suggest a possible basis for the diminished physiological potency of the acetic and propionic acid analogues in the rat.

IT HAS been demonstrated repeatedly that relatively minor changes in the structure of the thyroxine molecule result in a profound alteration in physiological activity *in vivo* (1). Thus, 3,5,3′-L-triiodothyronine (triiodothyronine) is more effective than L-thyroxine (thyroxine) by most *in vivo* assays, whereas analogues with a modified side chain (e.g., D-thyroxine, the acetic and propionic acid analogues) are less potent (cf. 1). In contrast with these findings *in vivo*, in most *in vitro* test systems these analogues have a potency approximately equal to that of thyroxine; for example, they are equally effective in "uncoupling" oxidative phosphorylation and in altering the morphology of mitochondria (2, 3, 4).

It appears possible that the disparity between the *in vitro* and *in vivo* findings may be explained by the fact that these various analogues are distributed to the tissues *in vivo* in quite different concentrations (5–7). It has been shown previously that triiodothyronine leaves the intravascular compartment very rapidly and reaches concentrations relatively higher than those of L-thyroxine in the peripheral tissues (cf. 5, 7) and it seems reasonable to speculate that the greater potency of triiodothyronine *in vivo*

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may be due to its greater concentration in these tissues. Likewise it has been demonstrated that there is a marked difference in the physiological disposition of labeled D- and L-thyroxine (6). Thus, the concentration of L-thyroxine in rat muscle is as much as six times greater than that of D-thyroxine; in the brain the difference is as great as 14-fold. In view of these major changes in distribution resulting from an alteration in the position of the amino group relative to the beta carbon of the side chain, the present study was undertaken to determine the effect of removing this group from the side chain, utilizing as examples of this the acetic and propionic acid analogues of thyroxine and of triiodothyronine.

MATERIALS AND METHODS

Male rats of the Wistar strain weighing between 100 and 200 gm. were used for all experiments; they were allowed Purina diet and tap water ad libitum. L-thyroxine, tetraiodothyroacetic acid (Tetrac), tetraiodothyropropionic acid (Tetraprop) labeled in the 3′,5′ position with I^{131}, and 3,5,3′-L-triiodothyronine, 3,5,3′-triiodothyroacetic acid (Triac) and 3,5,3′-triiodothyropropionic acid (Triprop) labeled in the 3′ position with I^{131} were obtained from Abbott Laboratories, Oak Ridge, Tennessee. By paper chromatography (6) they contained 15%, 23%, 22%, 15%, 23% and 16% free iodide, respectively.

Under ether anesthesia, 0.41 μg. L-thyroxine (specific activity 16–29 mc./mg.), 0.3 μg. Tetrac (specific activity 30 mc./mg.), 0.38 μg. Tetraprop (specific activity 14 mc./mg.), 0.2–0.4 μg. triiodothyronine (specific activity 55 mc./mg.), 0.22 μg. Triac (specific activity 35 mc./mg.) or 0.4 μg. Triprop (specific activity 16 mc./mg.) in less than 0.5 ml. of normal saline was injected into the tail vein. Groups of 5 to 10 animals were used for each time interval; all animals in any one group were injected with an identical quantity of labelled hormone. At the appropriate time following injection, the animals were sacrificed and the tissues, urine and feces were collected, prepared and counted as described elsewhere (6). Results were calculated as:

1) Percentage of the total injected radioactivity in each tissue.
2) Percentage of the total injected radioactivity per gm. of each tissue.
3) $\dfrac{\text{Concentration (\% total radioactivity/gm.) in tissue}}{\text{Concentration (\% total radioactivity/ml.) in plasma}}$

At selected times, butanol-extractable radioactivity was determined in several tissues essentially as by Van Arsdel *et al.* (5). Liver, kidney and skeletal muscle were extracted in the following manner: A sample of each tissue was homogenized in a small quantity of distilled water, an amount of 30% trichloracetic acid (TCA) equal to one-half the homogenate volume was added immediately and the mixture again homogenized. After an aliquot of the TCA homogenate was taken for counting, the rest was centrifuged, washed with 5% TCA and an aliquot of the pooled supernatant counted. The precipitate was extracted three times with N-butanol and an aliquot of the pooled extract counted. Feces were extracted in the same way as tissues; blood was placed directly into 10% TCA without preliminary homogenization, and urine was extracted after addition of crystalline serum albumin followed by TCA precipitation. Correction factors for incomplete recovery were obtained by extracting tissues, feces and urine to which a known amount of hormone had been added *in vitro*. After correction for incomplete recovery (in no tissue except muscle did the loss exceed 10%) the percentage of activity present in the butanol extracts was considered representative of the per-

centage of hormone present in the original tissue (5). The extractions with muscle were unsatisfactory for three reasons: first, homogenization was difficult and probably less complete than with the other tissues; second, the supernatant following the addition of TCA was slightly cloudy; and third, counts in the extracts were never more than two to three times background.

In experiments designed to establish the identity of the butanol extractable radioactive material present in the tissues, animals were sacrificed at 1 and 3 hours following the intravenous injection of labelled compound. Aliquots of blood, liver, kidney and muscle were homogenized in 3 ml. of water, extracted first with 10 volumes of 3% NH_4OH in butanol and then with three volumes of butanol. Following extraction with an equal volume of water, the butanol was concentrated under reduced pressure to a volume of less than 1 ml. This material was then chromatographed both in tertiary amyl alcohol saturated with 2N NH_4OH and in butanol-acetic acid-water solvent. The quantity of radioactivity injected was such that adequate chromatography could not be performed with tissues obtained later than 3 hours after injection. All counts were corrected for decay of radioactivity when appropriate.

RESULTS

The relative distributions of triiodothyronine and thyroxine.—It has been reported previously that the distribution of triiodothyronine in the intact rat is quite different from that of thyroxine (5, 7). The findings in the present investigation confirm the earlier observations. Following intravenous injection, triiodothyronine left the intravascular compartment more rapidly than did thyroxine and was concentrated to a greater extent in such tissues as skeletal muscle, heart, brain, spleen, and the gastrointestinal tract. Triiodothyronine was also excreted more rapidly than was thyroxine; approximately 50% of the radioactivity was excreted within 18 hours following the injection of labeled triiodothyronine, and within 42 hours following the injection of labeled thyroxine.

For the purpose of clarity the data related to thyroxine analogues and those related to triiodothyronine analogues are presented separately in the following sections.

Distribution of thyroxine analogues in various whole tissues.—After one hour the total recovery of injected radioactivity was 90% with thyroxine, 77% with tetrac and 81% with tetraprop (Figs. 1 and 2). Approximately 80% of the radioactivity recovered at one hour was found in blood, liver, kidney, muscle and skin with all three compounds. More tetrac and tetraprop than thyroxine were still present in the intravascular compartment, as seen in Figure 1. On the other hand, less Tetrac and Tetraprop than thyroxine were present in liver, kidney, skeletal muscle, skin, fat, brain, spleen, adrenal, intestine, lung and bone. The distribution of the three compounds in blood, liver, kidney, skeletal muscle, heart, brain and skin was determined at various intervals up to 72 hours after injection; the results are presented in Figure 3. Radioactivity disappeared less rapidly from blood and plasma following injection of the two analogues than it did following the injection of thyroxine; the differences were highly significant (P

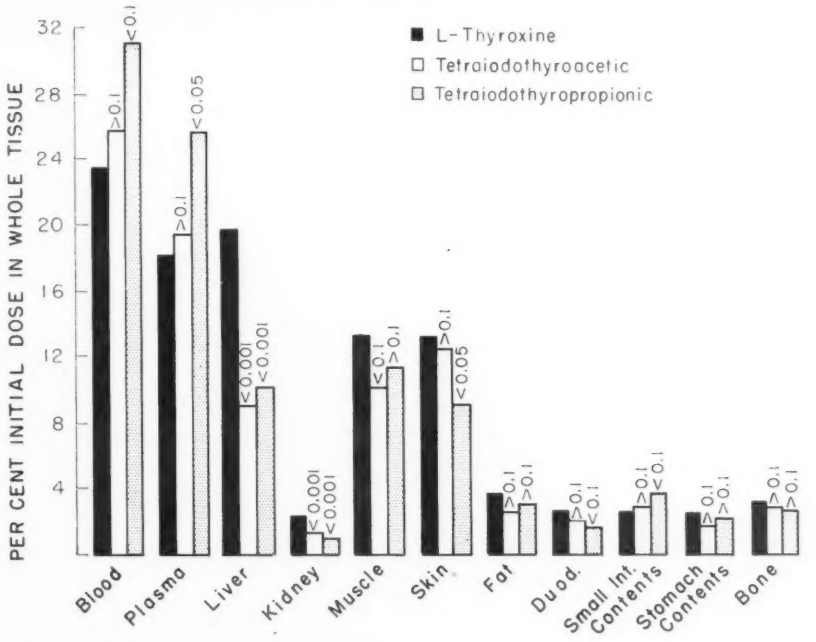

FIG. 1. Percentage of injected radioactivity in various whole tissues one hour following the injection of thyroxine, Tetrac or Tetraprop. (Statistical significance of the differences is indicated by the values for P obtained by using Student's t test.)

<0.001) at all time intervals except one hour. In contrast, the quantity of Tetrac and Tetraprop in the liver and kidney was significantly less ($P < 0.001$) than that of thyroxine for 18 hours after injection. The most strik-

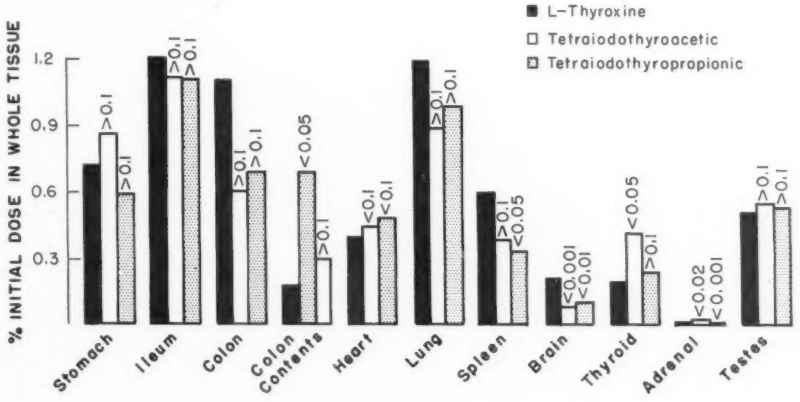

FIG. 2. Percentage of injected radioactivity in various whole tissues one hour following the injection of thyroxine, Tetrac or Tetraprop.

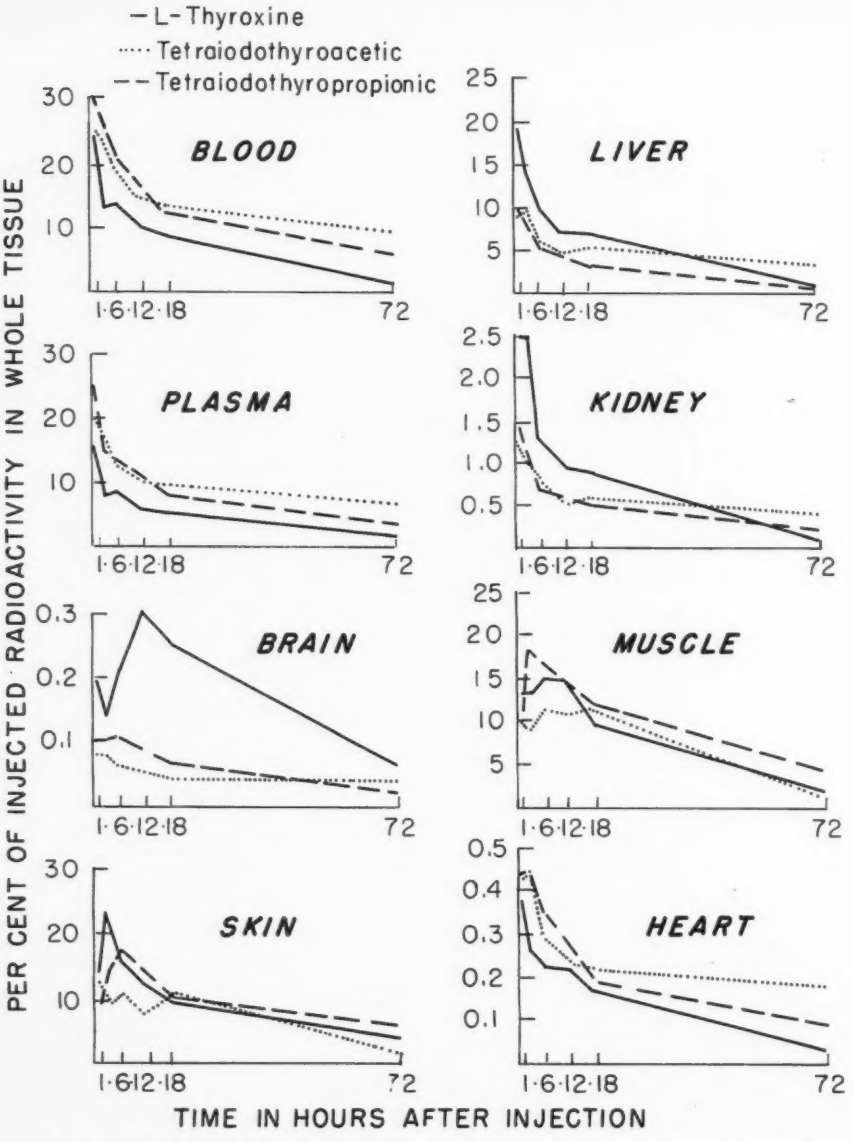

FIG. 3. Percentage of initial dose in various whole tissues as a function of time.

ing difference occurred in the brain, where the amount of thyroxine was six times greater than that of the two analogues. Differences observed in muscle and skin were slight, and in no case were they highly significant. The quantity of Tetrac and of Tetraprop in heart muscle was significantly

greater than that of thyroxine at all time intervals except 12 and 18 hours. At no time interval and in none of the tissues were the differences between Tetrac and Tetraprop statistically significant.

Concentration of thyroxine analogues in various tissues.—The percentage of the total radioactivity per gm. of each tissue one hour following injection is shown in Figure 4. With all three compounds the tissues with the greatest uptake per unit weight were liver, kidney, lung and pituitary; in liver and kidney the concentration of the two analogues was significantly less than that of thyroxine. The actual concentration of radioactivity in muscle and skin was low, although each of these tissues accounted for a large proportion of the total radioactivity because of its relatively great mass.

The percentage of the injected radioactivity per gm. (or ml.) of blood, plasma, liver, kidney, heart, skeletal muscle, brain and skin at various times following injection is presented in Figure 5. The concentration of the two analogues was significantly greater ($P < 0.001$) than that of thyroxine in blood and plasma at all time intervals after one hour. On the other hand, the concentration of the two analogues was significantly less ($P < 0.01$)

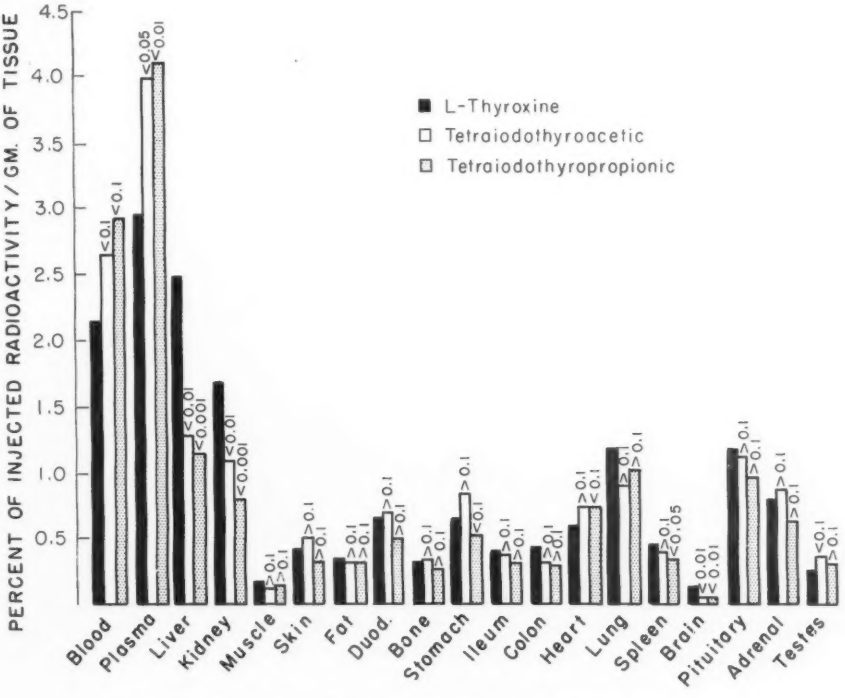

FIG. 4. Percentage of injected radioactivity per gm. of each tissue one hour following injection.

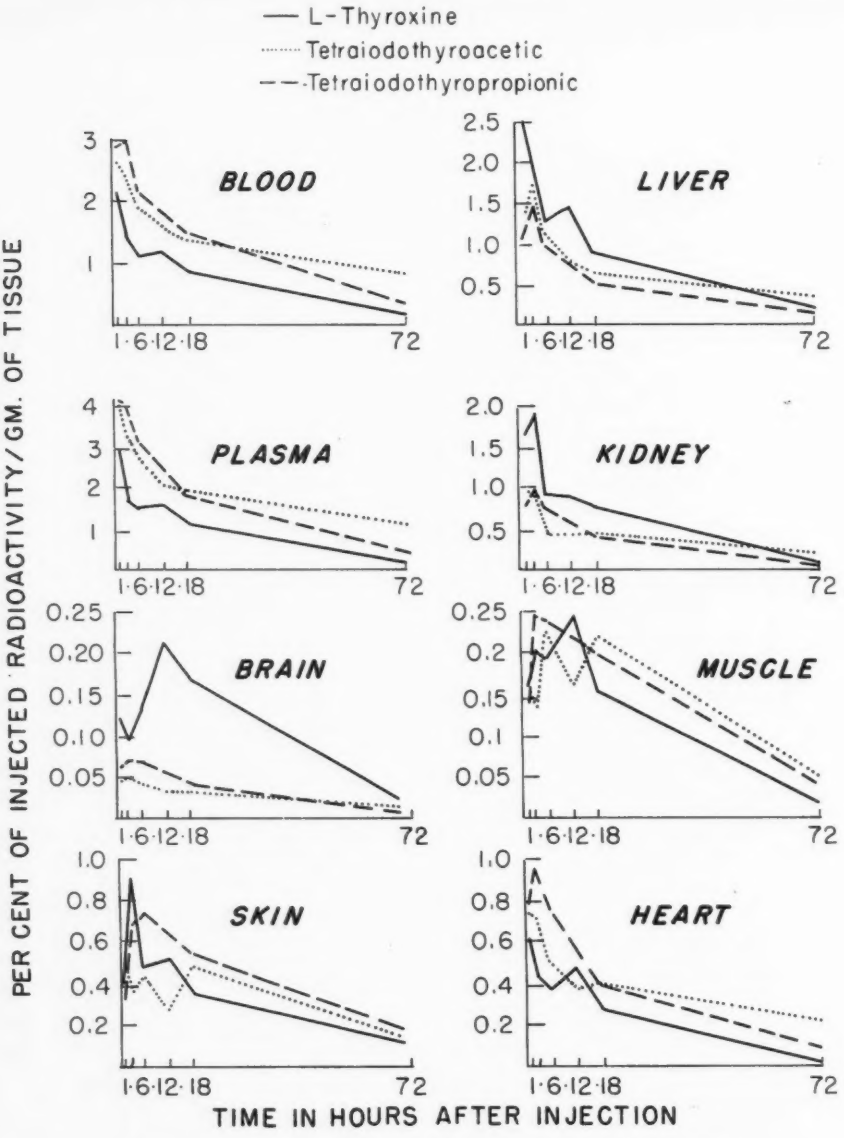

FIG. 5. Percentage of injected radioactivity per gm. of various tissues as a function of time.

than that of thyroxine in both liver and kidney except at 6 and 72 hours. The greatest difference noted was in the brain, where the concentration of thyroxine was as much as five times that of Tetrac and Tetraprop. The

concentration of thyroxine in heart muscle tended to be less than that of the analogues, but only at 3 and 6 hours was the difference highly significant ($P < 0.001$). The concentration of all three compounds in muscle and skin was approximately the same at all time intervals. Thus it is apparent that Tetrac and Tetraprop were removed more slowly than thyroxine from the intravascular compartment following intravenous injection, and that heart muscle was the only tissue in which they reached a higher concentration than did thyroxine.

Tissue/plasma ratios with thyroxine analogues.—If one considers only the absolute concentration of the compounds in the various tissues, as presented in Figures 4 and 5, no consistent difference is apparent between thyroxine and the two analogues. The relative concentration of thyroxine was greater in liver, kidney and brain, less in heart, and approximately the same in muscle and skin. However, the ratio

$$\frac{\%\text{ of total radioactivity/gm. tissue}}{\%\text{ of total radioactivity/ml. plasma}}$$

more accurately represents the affinity of the various tissues for the circulating compounds. Tissue/plasma ratios at each time interval for liver, kidney, muscle, heart, skin and brain are plotted in Figure 6. It may be seen that the tissue/plasma ratios at all time intervals and in all tissues were greater with thyroxine than with either Tetrac or Tetraprop. From the data presented in Figure 4 it may be calculated that the tissue/plasma ratios in all other tissues examined at one hour were likewise greater with thyroxine than with the two analogues.

Excretion of radioactivity in urine and feces (thyroxine analogues).—The total excretion of radioactivity 72 hours following the injection of all three compounds fell within the range of 60 to 70%. In each case approximately one-half of this excreted radioactivity was in the urine and one-half in the feces. In the urine, most of this radioactivity (91%, 99% and 98% with thyroxine, tetraprop and tetrac respectively) was in the form of iodide. In the feces the reverse was true; approximately 10% of the radioactivity was iodide with all three compounds; the remainder was butanol-extractable and thus presumably hormonal.

Distribution of triiodothyronine analogues in various whole tissues.—The total recovery of radioactivity one hour following injection (Figs. 7 and 8) was 92% with L-triiodothyronine, 84% with Triac and 82% with Triprop. Of the injected radioactivity recovered at one hour, that in blood, liver, kidney, muscle and skin represented approximately 70% of the total with triiodothyronine and 60% with both Triac and Triprop. The total quantity of radioactivity in the blood was greater with the two analogues than with triiodothyronine; on the other hand, in liver, kidney, skeletal muscle, heart, brain and spleen the total quantity of the two analogues was less than that of tiiodothyronine.

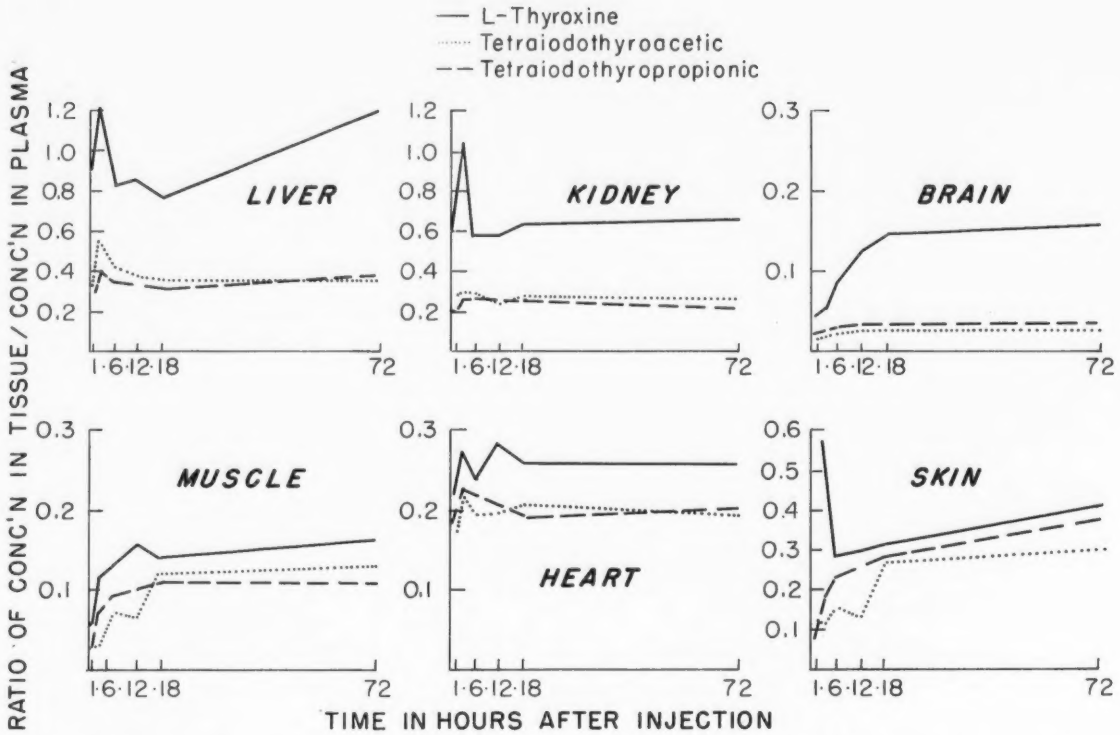

FIG. 6. Ratio of concentration of radioactivity in certain tissues to the concentration in plasma at various times after the injection of labeled hormone.

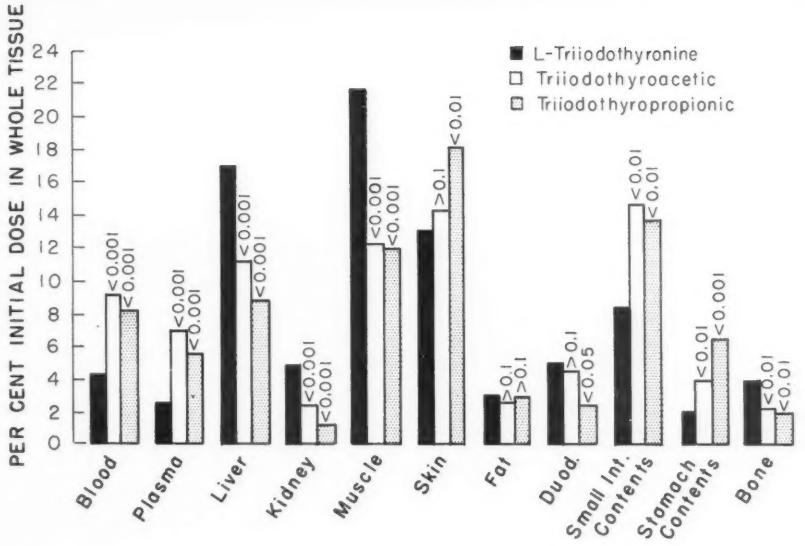

FIG. 7. Percentage of injected radioactivity in various whole tissues one hour following the injection of triiodothyronine, Triac or Triprop. (Statistical significance of the differences is indicated by the value for P obtained by using Student's t test.)

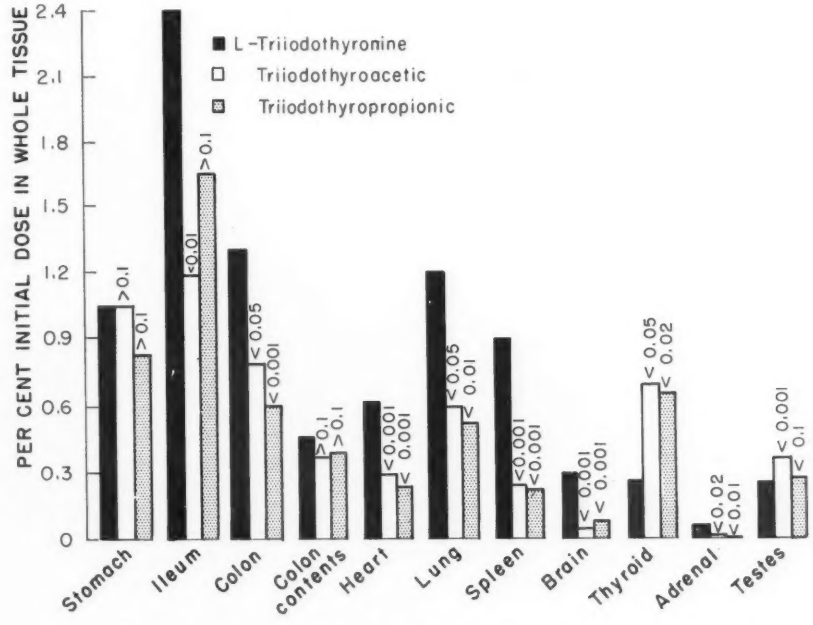

FIG. 8. Percentage of injected radioactivity in various whole tissues one hour following the injection of triiodothyronine, Triac or Triprop.

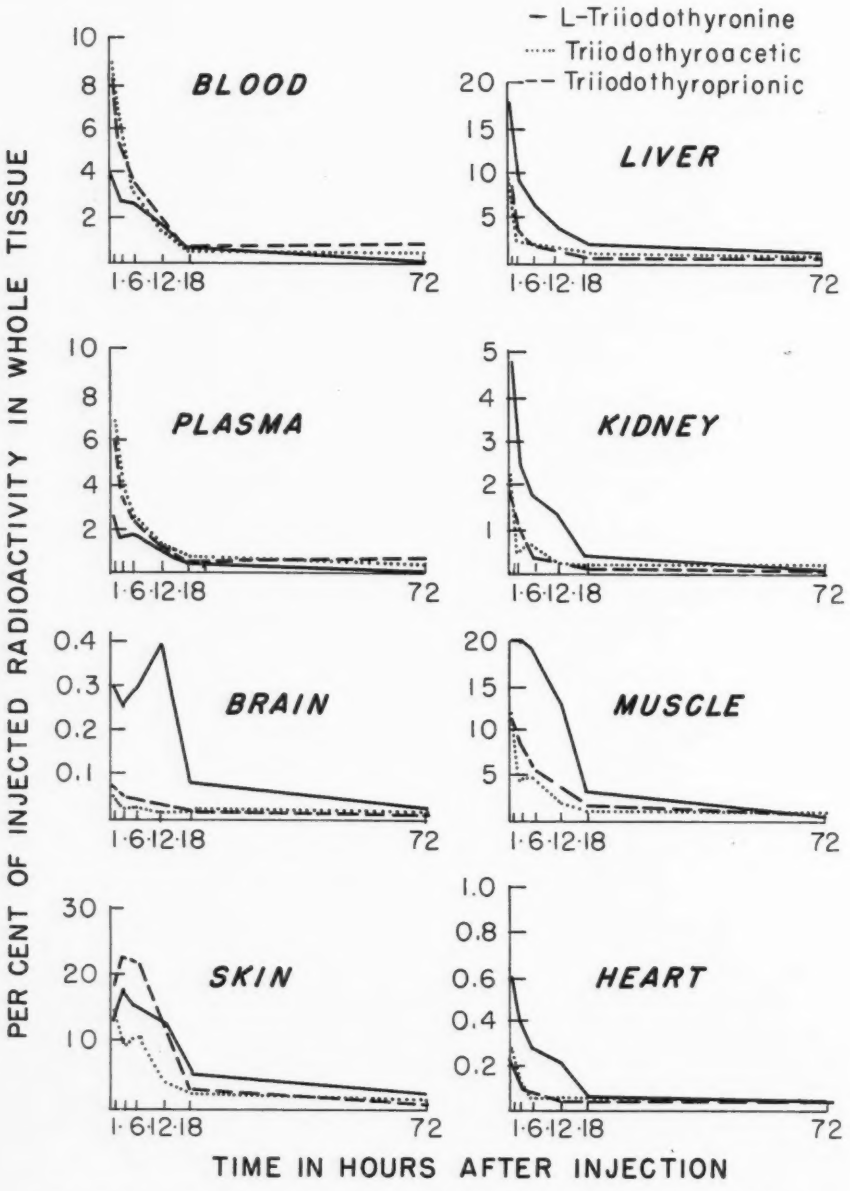

FIG. 9. Percentage of initial dose in various whole tissues as a function of time.

The distribution of the three compounds in blood, plasma, liver, kidney, skeletal muscle, heart, brain and skin was determined at various intervals up to 72 hours following injection. As can be seen in Figure 9, radioactivity disappeared less rapidly from blood and plasma following injection of the two analogues than following the injection of triiodothyronine; the amount of Triac and Triprop still in the blood was significantly greater ($P < 0.001$) for the first six hours. By contrast, the quantity of Triac and Triprop in liver, kidney, brain, muscle and heart was significantly less ($P < 0.001$) than that of triiodothyronine up to 12 hours after injection. It is apparent from Figure 9 that the most striking difference between triiodothyronine and the two analogues occurred in the brain. In muscle the differences were such that at 12 hours there was approximately 4 to 5 times more triiodothyronine than Triac or Triprop. The differences observed in skin were not significant. At no time interval and in none of the tissues were the differences between triac and triprop statistically significant.

The concentration of triiodothyronine analogues in various tissues.—The percentage of the total radioactivity per gm. of each tissue one hour following injection is shown in Figure 10. The concentration of Triac and Triprop was significantly less than that of triiodothyronine in all tissues examined except stomach, duodenum, ileum, testes, fat and skin; con-

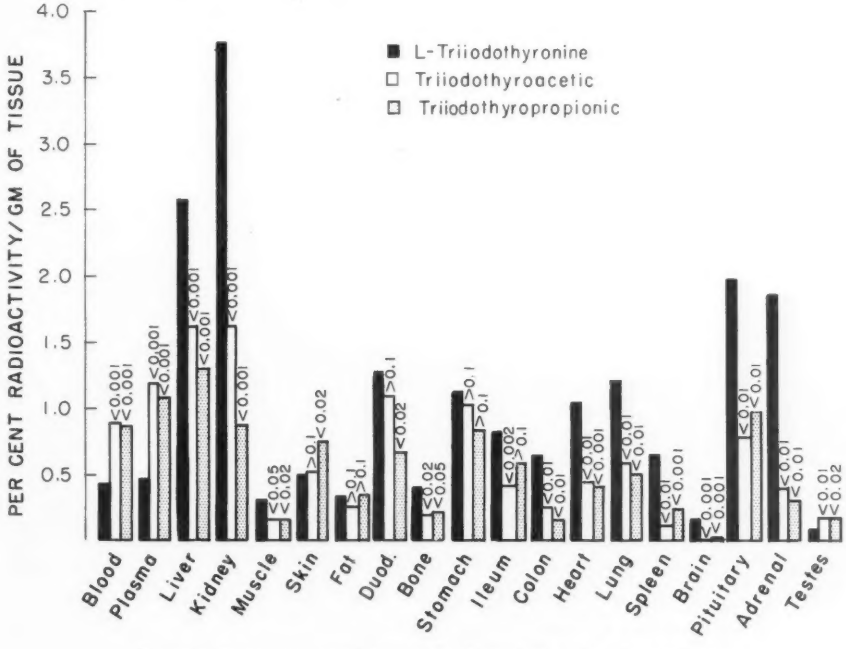

FIG. 10. Percentage of injected radioactivity per gm. of each tissue one hour following injection.

versely, the concentration of Triac and Triprop in the blood was significantly greater than that of triiodothyronine.

The percentage of the injected radioactivity per gm. (or ml.) of blood, plasma, liver, kidney, heart, skeletal muscle, brain and skin at various times following injection is presented in Figure 11. The concentration of the two analogues was significantly greater ($P < 0.001$) than that of triiodothyronine in blood and plasma for the first six hours. On the other hand, the concentration of the two analogues was significantly less ($P < 0.001$) than that of triiodothyronine for the first 12 hours in liver, kidney, brain, skeletal muscle and heart. The greatest differences were observed in brain, muscle and heart; the differences in skin were not significant at any time interval. Thus, following intravenous injection, Triac and Triprop were removed more slowly than triiodothyronine from the intravascular compartment and picked up to a lesser extent by the peripheral tissues.

Tissue/plasma ratios with triiodothyronine analogues.—Tissue/plasma ratios at each time interval for liver, kidney, muscle, heart, skin and brain are plotted in Figure 12. It is apparent that the tissue/plasma ratios at each time interval and in all tissues were greater with triiodothyronine than with either Triac or Triprop. From the data presented in Figure 10, it may be calculated that the tissue/plasma ratios with all the other tissues examined at one hour were likewise greater with triiodothyronine than with either Triac or Triprop.

Excretion of radioactivity in urine and feces (triiodothyronine analogues).—Total excretion of radioactivity 72 hours following the injection was 80 to 90 % with all three compounds. In each case roughly one-half of the excreted radioactivity was in the urine and one-half in the feces. With all three compounds approximately 98% of the radioactivity in the urine was in the form of iodide. In the feces the reverse was true; 80 to 90% of the radioactivity was butanol-extractable and thus presumably hormonal.

Identification of radioactivity in tissues (thyroxine and triiodothyronine analogues).—Butanol extractable radioactivity of TCA-precipitates (i.e., radioactivity not in the form of free iodide) was determined in blood, liver, kidney and muscle, one and three hours following injection. With thyroxine approximately 90% of the radioactivity in blood was butanol-extractable; with Tetrac and Tetraprop 98% and 97%, respectively, were in this form. In liver and kidney, the percentage of butanol-extractable radioactivity was approximately the same (90 to 95%) with all three compounds. Following injection of triiodothyronine, approximately 65% of the radioactivity in the blood was butanol-extractable; with both Triac and Triprop 55 to 60% was butanol-extractable. In both liver and kidney, the butanol-extractable radioactivity with triiodothyronine amounted to 85 to 90% of the total, with Triac 65 to 70% and with Triprop 50 to 55%. For many reasons (6) the extractions with muscle were unsatisfactory and the data are considered unsuitable for presentation.

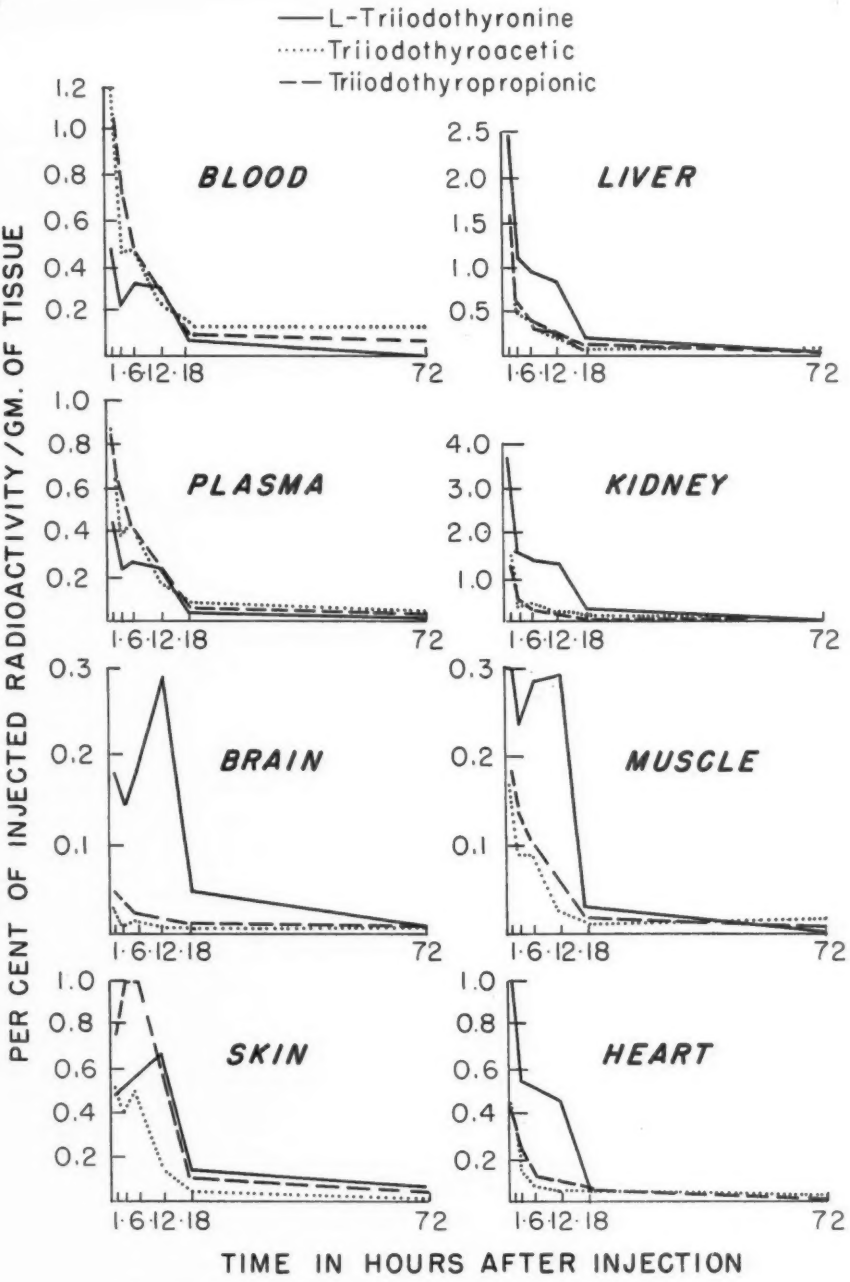

FIG. 11. Percentage of injected radioactivity per gm. of various tissues as a function of time.

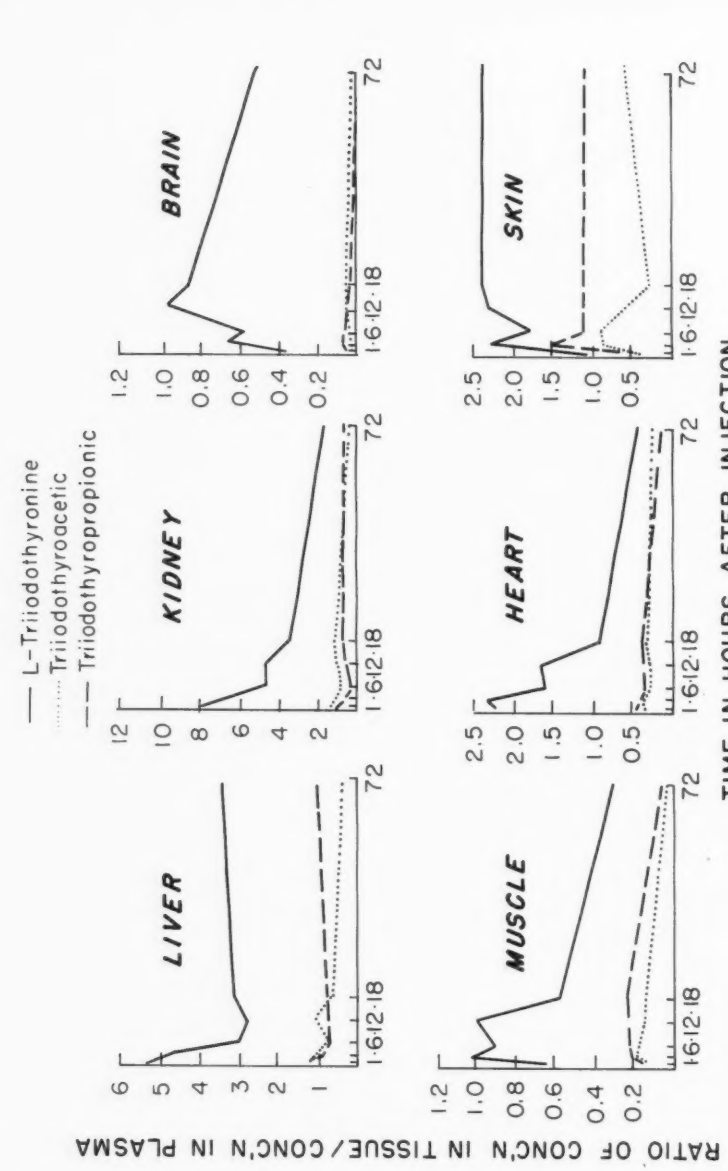

FIG. 12. Ratio of concentration of radioactivity in certain tissues to the concentration in plasma at various times after the injection of labeled hormone.

In separate experiments, direct butanol extracts of blood, liver, kidney and skeletal muscle (tissues obtained both one and three hours following injection) were concentrated to a volume of less than 1 ml. and the materials chromatographed in both tertiary amyl alcohol saturated with ammonia and in butanol-acetic acid-water solvent. With all six compounds and in both solvent systems all the radioactivity had an Rf similar either to that of the injected compound or to iodide. However, the quantity of radioactivity recovered in these extractions was so low that any degradation product would have had to contain 5 to 10% of the butanol-extractable radioactivity to be detectable.

DISCUSSION

The distribution of L-thyroxine, L-triiodothyronine and of Triac reported in this study is similar to that observed by others (5, 7); the distribution of Tetrac, Tetraprop and Triprop has not been studied previously. Although the quantity of labeled hormone injected in this investigation represented less than 20% of the estimated daily output of thyroxine from the normal rat thyroid (8), it was undoubtedly considerably in excess of the quantity of the other injected compounds present in the normal rat. Furthermore, there is no evidence that Tetrac, Triac, Tetraprop or Triprop are secreted by the thyroid and distributed to the tissues via the circulation. Under normal conditions the small amounts of these compounds which are present are presumably produced in the peripheral tissues (9). These studies should not be interpreted, therefore, as bearing any relationship to the usual physiological disposition of these compounds as they are formed in the peripheral tissues. Since the thyroids were not removed from these animals, some of the radioactivity present at the later time intervals must have been in the form of newly synthesized thyroxine. Unfortunately the quantity of radioactivity which could be recovered at intervals later than 3 hours was not sufficient to allow for chromatographic identification. At 3 hours only the injected compound could be identified in the tissues.

It is difficult to judge accurately from the literature the relative potency of these compounds. In general, it appears that both Tetrac and Tetraprop are less active *in vivo* than is thyroxine, but considerable variation has been observed by different investigators and with different assay procedures. Early work suggested that Tetrac was active *in vitro* and *in vivo* immediately (10–14), but this has not been confirmed (15–18). In the rat Tetrac has been found to have 2 to 57% of thyroxine activity by goiter-prevention assay (11, 18), 11% by calorigenic assay (18), 70 to 75% by *in vitro* assay of tissue oxygen consumption following injection into the whole animal (19). Tetraprop has been found to have 14 to 75% of thyroxine activity by goiter-prevention assay (18, 20–22), 7 to 10% by calorigenic assay (18, 20, 21), 75% by *in vitro* assay of tissue oxygen consumption following injection into the whole animal (17, 23) and 60% by assay of the suppression of I^{131}

uptake by the thyroid (19). In marked contrast to these findings in mammalian assays, the effect of Tetraprop on tadpole metamorphosis is 21 to 65 times that of thyroxine (21, 24, 25). Certainly there is general agreement that triiodothyronine exerts its metabolic effect more rapidly than does thyroxine (1). There is almost general agreement that Triac and Triprop are less effective than triiodothyronine and probably less effective than thyroxine. In the rat, Triac has been found to have 50 to 75% of thyroxine activity by goiter-prevention assay (11, 18), 20 to 75% by calorigenic assay (11, 18) and 75% by assay of the suppression of I^{131} uptake by the thyroid (19). Triprop has been found to have 30 to 75% of thyroxine activity by goiter-prevention assay (18, 20, 21), 10 to 15% by calorigenic assay (18, 21) and 60% by assay of the suppression of I^{131} uptake by the thyroid (19). The present study, showing that thyroxine and triiodothyronine leave the intravascular compartment and enter the tissues more readily than their respective acetic and propionic acid analogues, suggests a possible explanation for the lesser potency of the analogues *in vivo* in the rat. It may be noted that there was no unusual distribution of the acetic acid analogues which might be expected if the time of onset of action of these compounds were indeed immediate (10–14). However, it should be emphasized that the differences in distribution noted in this study may not occur in other species.

These differences in distribution appear to be dependent upon a different capacity of the tissues to bind the various compounds. As indicated by the tissue/plasma ratios, the affinity of the tissues for the acetic and propionic acid analogues is less than that for their parent compounds. *In vitro* studies (unpublished data) indicate that the uptake of these compounds by various tissues (liver, kidney, skeletal muscle, heart, spleen, brain, stomach, colon, lung and fat) is not an active one (i.e., it is not dependent upon oxidative processes) but is apparently caused by binding. In a medium containing no protein, thyroxine, Tetrac, Tetraprop, Triac and Triprop are bound to all of the above tissues to approximately the same extent; triiodothyronine is bound to a slightly greater extent. The situation *in vivo* is obviously more complex. It must depend upon the relative binding capacity both of the tissues and of the plasma proteins as well as upon the relative rates at which these compounds are degraded in various tissues, excreted in the bile and urine and reabsorbed from the gut.

It is of interest to compare the effect produced by removing the amino group from the thyroxine molecule (as in Tetrac and Tetraprop) with the effect produced by changing the relative position of the amino group (as in D-thyroxine). Removal of the amino group leads to a decreased affinity for all tissues, as indicated by the decreased tissue/plasma ratios for Tetrac and Tetraprop. A change in the relative position of the amino group, on the other hand, results in an increased affinity for liver and kidney (as revealed by greater tissue/plasma ratios for D-thyroxine) but in no signifi-

cant change in the affinity for other tissues (6). Removal of the amino group from triiodothyronine results in an alteration in its distribution similar to that produced with removal of the amino group from thyroxine. Finally, the distribution of Tetrac is essentially the same as that of Tetraprop and the distribution of Triac essentially the same as that of Triprop; it seems reasonable to conclude, therefore, that it is the removal of the amino group which leads to changes observed and that the additional removal of a carbon atom from the side chain does not further alter physiological disposition.

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INDUCTION OF THYROTROPIN-DEPENDENT THYROID TUMORS BY THYROTROPES[1]

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ABSTRACT

Transplantable thyroid tumors were induced by grafting thyroids of mice with an autonomous but functional thyrotropic tumor. The latter provided the necessary stimulus of sustained high levels of thyrotropin.

These thyroid tumors remained fully dependent after four consecutive animal passages, i.e., they failed to grow in the absence of a thyrotropic tumor.

Although remaining fully dependent in the course of four passages, the tumors gradually assumed the histological appearances associated with malignant growth, as manifested by cellular pleomorphism, loss of polarity, and frequency of mitotic figures. The tumor appeared to be locally invasive, but there was no evidence of distant metastases. The latency of the tumors was long (8–10 months) when the thyroid and thyrotropic tumors were grafted simultaneously. It could be reduced greatly by grafting the thyrotropic tumor much before the thyroid tumor.

HYPERPLASTIC lesions have been induced in the thyroids of rats and mice by the long continued administration of antithyroid drugs, as brassica seeds (1), thiourea (2), thiouracil (3–5), and propylthiouracil (6); and by radioiodine (7–10) and low iodine diet (11). These lesions range from simple hyperplasia through adenomas to adenocarcinoma, in some instances with metastases. Initially these thyroid tumors could be transplanted only to animals similarly treated (12), but on repeated passages they gave rise to autonomous variants (13–16).

Interference with thyroid pituitary hormonal balance may result in the development of either pituitary or thyroid tumors (17). In particular, destruction of the thyroids of mice by I^{131} has resulted in the production of pituitary tumors (18). These tumore secrete thyrotropin and are transplantable (19). Although initially dependent, i.e., growing only in the athyroid animal, they become autonomous (20) in the course of successive passages, i.e., they grow in euthyroid hosts.

The animals bearing these transplanted autonomous thyrotropic tumors have greatly enlarged thyroids. The thyroid glands are hyperplastic, con-

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taining many adenomas and occasionally areas suggestive of carcinoma. Nevertheless attempts to transplant these thyroid tumors have been unsuccessful until the development of tumors here described.

MATERIALS AND METHODS

Female LAF_1 mice, obtained from the Roscoe B. Jackson Memorial Laboratory, were used. They were fed Purina chow and given tap water *ad libitum*. At the time of grafting they were aged 7–9 weeks.

For initial transplantation hyperplastic adenomatous thyroids were used, and in the subsequent passages tumors resulting from them. In the earlier passages the tumors measured only about 5 mm., and this necessitated the pooling of several tumors. The tumors were minced with scissors in sterile normal saline and 0.05–0.1 ml. of an approximately 20% tumor mince was injected intramuscularly in the thigh. For histological examination tissues were fixed in Formol-Zenker, sectioned at 6 μ and stained with hematoxylin and eosin.

The thyroid grafts were made in both normal and radiothyroidectomized animals. For radiothyroidectomy mice were kept for 10 to 20 days on a low iodine diet (obtained from Nutritional Biochemicals Corp.) and were given 60 μc. of I^{131} subcutaneously. In some animals a hypothyroid state was induced by the administration of propylthiouracil (PTU) in drinking water (0.02%), given *ad libitum*, supplemented by desiccated thyroid powder in the food (0.1%). Some animals were also grafted in the other thigh with thyrotropic pituitary tumors (TtT) of a slowly growing autonomous but functional strain.

RESULTS

After the original passage, the grafted tumors were carried in two separate lines (8 & 34, Table 1). These behaved essentially alike. The thyroid tumors grew very slowly in normal mice bearing TtT and, for reasons unknown, even more slowly in athyroid mice bearing TtT. To date four con-

TABLE 1. RESULTS OF TRANSPLANTATION OF THYROID TUMORS IN IN NORMAL AND CONDITIONED HOSTS

Line	Passage	Time of TtT graft	Treatment of host				
			Bearing TtT		Without TtT		
			Normal	Athyroid	Normal	Athyroid	PTU treated
34	I	Simultaneous	3/4 (270)*		0/5		
	II	Simultaneous	5/6 (250)	3/5 (310)	0/5	0/5	
	IIIA	90 days earlier	8/8 (60)		0/8		
	IIIB	Simultaneous	7/8 (218)	2/8 (282)	0/8	0/8	
	IV	60–130 days earlier	2/6 (220)	5/9 (36)	0/13	0/13	
Total			25/32	10/22	0/39	0/26	
8	I	Simultaneous	2/3 (325)		0/6	0/6	
	II	109 days later	5/6 (326)	2/4 (376)			0/9
Total			7/9	2/4	0/6	0/6	0/9

* Number of animals with tumors over number grafted. Average latency (i.e., time when the tumor became palpable) in days, is given in parentheses.

Many animals in Passage IV are still alive.

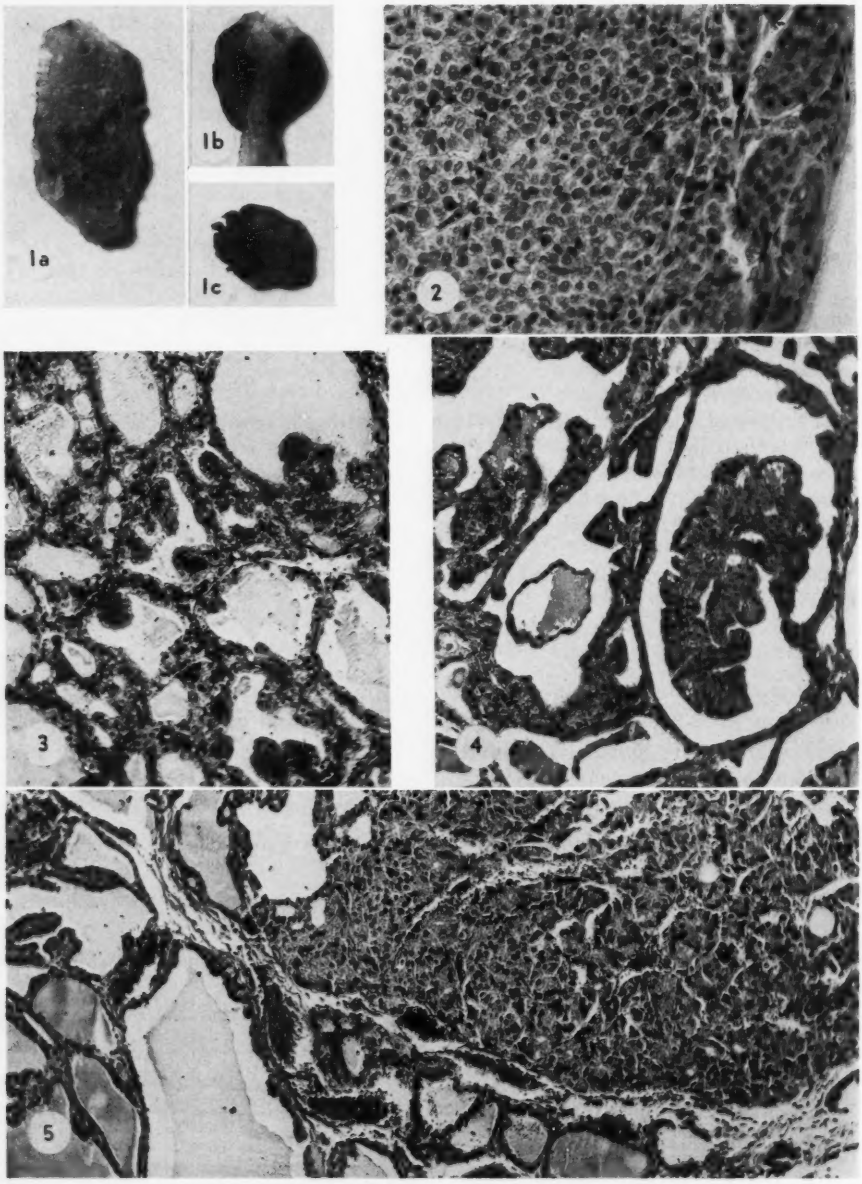

FIG. 1a. A transplantable thyroid tumor grown under the stimulation of thyrotropin. Note the resemblance to a colloid goiter. (×2.5)
FIG. 1b. A hyperplastic thyroid stimulated by an intramuscular graft of a thyrotropic pituitary tumor. (×2.5)
FIG. 1c. The thyrotropic pituitary tumor which was the source of thyrotropin (×2.5)

secutive passages have been made in TtT treated animals. Grafts failed to take in animals not treated with TtT, and in PTU treated mice.

Tumor growth could be greatly hastened by building up a very high thyrotropin level by grafting prospective recipients with autonomous thyrotropic tumors weeks or months before grafting the thyroid tumor. This is illustrated in passage III A and passage IV in which the thyroid tumors became palpable in 60 and 36 days in animals pretreated with TtT.

In line 8, in which the latency periods were long (325–376 days), TtT was implanted 109 days after the thyroid tumor grafts. (The "dormancy" of these tumor cells in histocompatible hosts is well known.)

Anatomic characteristics. Figure 1 shows a grafted thyroid tumor (1a) and the greatly enlarged thyroid (1b) from an animal bearing a thyrotropic tumor (1c). The characteristics of the latter have been previously described (21–22) and its histological appearance is shown in Figure 2.

The enlargement of the thyroid in an animal having a thyrotropic tumor was symmetrical. Diffuse hyperplasia was followed by the development of adenomatoid nodules.

The microscopic changes in the evolution of thyroid tumors are illustrated in Figures 3–5. At first there was widespread hyperplasia followed by papillary infolding, progressing to adenomas, both papillary and solid. Normal, hyperplastic and tumorous areas occurred side by side. In general, the epithelial cells in the papillary areas were cuboidal with large central nuclei and granular eosinophilic cytoplasm. In the solid areas they were smaller and their nuclei were hyperchromatic. Pleomorphism, in general, was slight, and mitotic figures were few. In places pleomorphism, loss of polarity, and the many mitotic figures suggested an early malignancy. However, in the present series gross and microscopic examination failed to disclose the presence of thyroid tissue in the adjacent structures of the neck, lymph nodes and lungs.

The transplanted tumors were lobulated, reddish grey, and moderately firm. The gross appearance was reminiscent of a colloid goiter (Fig. 1a). Histologically (Figs. 6–9) the thyroid origin of the tumors was usually evident, there being well developed acini throughout, lined by cuboidal epithelium and containing colloid (Fig. 6). There were both solid and papillary areas (Fig. 7). The histological appearances showed more cellularity and

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FIG. 2. The histological appearance of the tumor shown above. Note the minimal degree of dedifferentiation. (×160)

FIG. 3. Thyroid stimulated by thyrotropic tumor. An early lesion with some papillary infolding. (×160)

FIG. 4. Thyroid stimulated by thyrotropic tumor. More advanced lesion showing papillary hyperplasia. (×160)

FIG. 5. Thyroid stimulated by thyrotropic tumor. A predominantly solid area. (×160)

less secretory activity than in the thyroid. There was more pronounced pleomorphism and mitotic figures were more frequent in both papillary and solid areas than in the thyroid (Fig. 9). Small nodules of thyroid tumor were present at some distance from the main tumor mass, where they were growing freely in the muscle (Fig. 8). However in none of the animals were distant metastases detected.

DISCUSSION

Dependent tumors of endocrine organs are of special interest; they provide "pure cultures" of functional cells which are fully under hormonal control and, therefore, a means of studying feedback regulation of the respective system.

Thyroid tumors have been induced by the administration of a variety of anti-thyroid substances. The postulated common mechanism of tumor induction is an upset of thyroid function leading to a deficiency of thyroid hormone. This in turn produces an increased secretion of thyrotropin which stimulates the thyroid and, in time, hyperplasia is followed by tumor formation.

In this series the sustained stimulation of the gland was produced by thyrotropin secreted by grafted autonomous thyrotropic tumor, which gave a higher thyrotropin blood level than other procedures (21). The thyroid changes in thyrotropic tumor-bearing animals were specific: they have not been observed in animals with other types of pituitary tumors of which adrenotropes and mammo-somatotropes have been studied extensively (20). The thyrotropin content of thyrotropic tumors has been assayed earlier (22). Dependent tumors had as high as 40 U.S.P. units per gm. wet weight. Autonomous functional tumors, as used in these experiments, have a lower thyrotropin level (12–15 U.S.P. units/gm. wet weight; assays of V. B. Kamat, to be published).

It follows that the growth stimulus of the thyroid tumors is thyrotropin derived from the thyrotropic tumors. The growth rate of stimulated thyroid tumor cells appears to be much greater than that of the animal's normal thyroid. These thyroid tumors induced by thyrotropic tumors appear to develop in a manner similar to those induced by other means. The initial changes were those of hyperplasia, which progressed to the formation of adenomas. Finally, there was the development of areas which on morphological grounds may be interpreted as carcinomas.

In experimental animal tumors, as in similar human neoplasms, the histological diagnostic criteria of malignancy are often unreliable, as indicated by biological behavior (cf. 5). In addition to the cytological features associated with the diagnosis of malignancy, such as pleomorphism, abnormal staining properties and frequency of mitotic figures, two features have been used in the histological grading of experimental and human tumors: extension of tumor beyond the capsule and the presence of tumor cells in blood

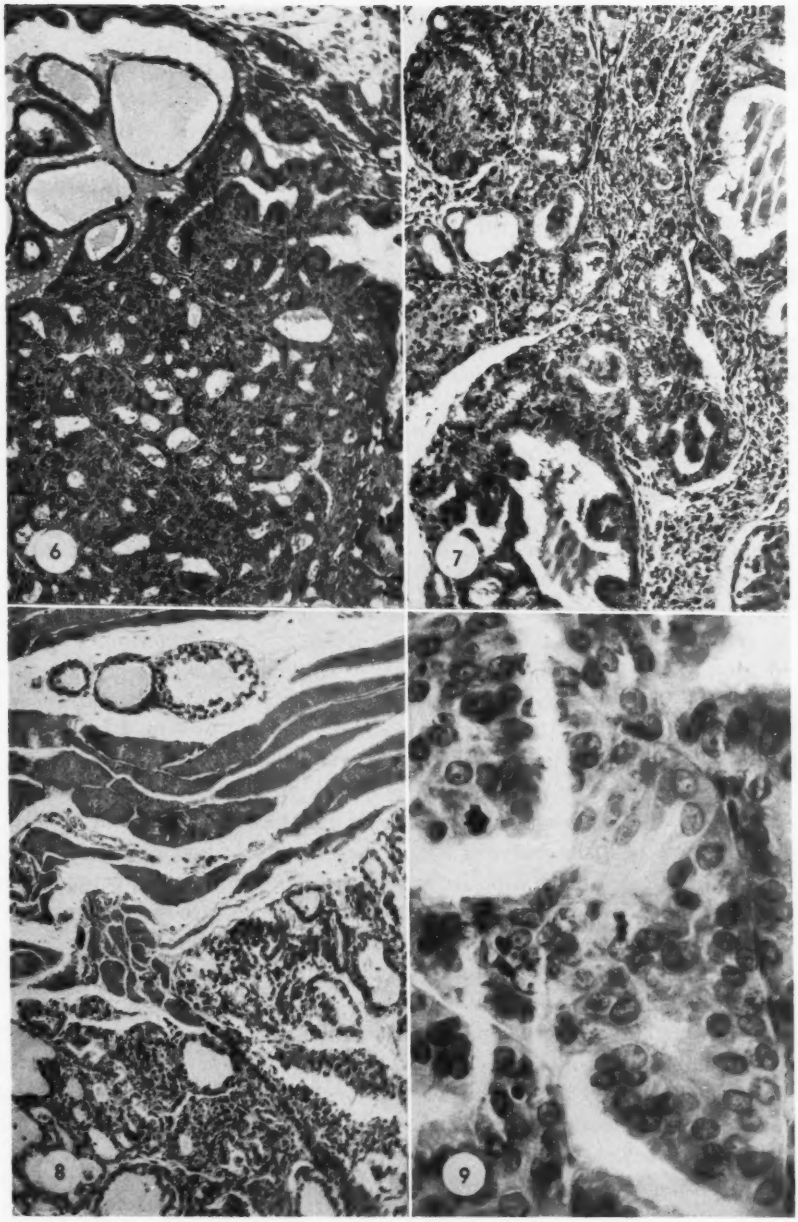

FIG. 6. Transplanted thyroid tumor showing small acini and solid areas. (×160)
FIG. 7. Transplanted thyroid tumor. Solid areas and papillary infolding. (×160)
FIG. 8. Transplanted thyroid tumor. Invasion of Muscle by acini containing colloid. (×160)
FIG. 9. Transplanted thyroid tumor. High power picture showing cellular pleomorphism and mitotic figures. (×625)

vessels. However, both of these features have been reported in "benign" tumors by Gorbman (23) who found hyperplastic thyroid tissue within the lumen of pulmonary vessels and in lung parenchyma of chronically thiouracil-treated mice. However, return to normal diet after 300–400 days on goitrogen diet was followed by involution of the supposedly cancerous thyroids. He considered that the hyperplastic thyroid tissue had entered the veins mechanically (*vis a tergo*) rather than by malignant infiltration, and that the changes were essentially benign. Similarly Dalton *et al.* (24) reported the presence of unencapsulated pulmonary metastases of thyroid tissue in C3H mice that had received thiouracil for 11 months. The thyroids of these mice showed only diffuse hyperplasia. Later, however, (15) when they established transplantable thyroid tumors which in time became autonomous, they revised their opinion, postulating that the "benign metastasizing thyroid" was potentially malignant.

Blood invasion by thyroid adenomas was also noted around thyroids stimulated by thyrotropic tumors (19). Transfer of these thyroids into normal hosts was unsuccessful, indicating lack of malignancy (autonomy). We consider that the so-called "benign metastasizing goiter" is a conditioned growth disturbance and not an autonomous neoplasm.

It is now generally recognized that both growth and function of the thyroid gland depend on the function of thyrotropes and that sustained excessive quantities of thyrotropin cause the development of thyroid tumors. Furthermore, the growth of these tumors which may by some standards be interpreted as "malignant" is also strongly influenced by the activity of thyrotropes. A similar state of affairs has been shown to exist in man, as indicated by the comprehensive review of Thomas (25). The study here reported shows that the grafts of autonomous highly functional thyrotropic tumors can induce and maintain thyroid tumors. The thyroid tumors so induced are dependent neoplasms, as shown by their failure to grow in the absence of the sustained stimulus of thyrotropin. In the light of experimental evidence, the controversy over the histological grading of the tumors is fruitless; what matters is the biologic grading into dependent and autonomous growth; and subdivision of the latter into hormone-responsive and fully autonomous neoplasms.

Treatment with PTU failed to support the growth of these thyroid tumors under the conditions of the experiment. The PTU diet was supplemented with desiccated thyroid as recommended by Sellers *et al.* (cf. 26). In their experience the goitrogenic effect can be enhanced by dried thyroid powder, administered concurrently over a specific range of dosage for long periods (8 to 18 months). Sellers and Schonbaum (26) mention several possibilities to explain this seemingly paradoxical phenomenon, but conclude that there is no convincing evidence supporting any one of them to the exclusion of the other. Thus the problem is unsolved. In our experiments this treatment caused goiter in all mice. Failure of this treatment to support the

growth of thyroid tumor may have been due to lower thyrotropin levels in mice so treated than in radiothyroidectomized mice.

Acknowledgment

We thank Dr. Kenjiro Yokoro for helping to complete the experiments here reported.

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EFFECT OF DIABETOGENIC HORMONES ON BLOOD SUGAR AND PANCREAS OF SULFONYLUREA TREATED ANIMALS[1]

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ABSTRACT

Studies have been conducted on the diabetogenic effectiveness of growth hormone in 50% partially pancreatectomized dogs and of cortisone in rabbits, which received tolbutamide prior to and during hormone administration. The degree of diabetes which was observed in each species was similar to that found in animals receiving hormones alone. Furthermore, the degree of B cell destruction was similar in dogs treated with growth hormone alone to that observed in those who received both the sulfonylureas and the growth hormone. This destruction of B cells has been interpreted to be a response to hyperglycemia through some unknown mechanism, but not due to functional stimulation of the B cells. This viewpoint was supported by the fact that chronic stimulation of the B cells by the sulfa drugs causes degranulation and enlargement of nuclei and Golgi apparatus but no other changes. Furthermore, pretreatment with sulfonylurea drugs did not measurably alter the pancreatic morphologic response of the rabbits or dogs to diabetogenic hormones.

IT IS generally believed that the administration of anterior pituitary extracts causes hyperglycemia in the dog by inhibition of peripheral utilization of glucose (1, 2). The resultant increased glycemic level stimulates insulinogenesis from the pancreatic B cells which is expressed morphologically as degranulation (3–8). The hypoglycemic sulfonylureas stimulate the B cells directly to increase insulin output and similarly cause degranulation (9–13). In previous studies the authors showed that idiohypophyseal diabetic dogs with complete degranulation of the pancreatic B cells did not develop a decline of the blood sugar after a single test dose of tolbutamide. However, the drug did evoke a hypoglycemic response in diabetic dogs with even minimal residual pancreatic B cell granulation (13). Moreover, in dogs with metahypophyseal diabetes and B cell destruction the daily administration of tolbutamide failed to lower the blood sugar level. Since complete degranulation or destruction of B cells are indicative of loss of pancreatic insulinogenic reserve these observations would indicate that some B cell functional reserve must be present for the hypoglycemic action of the sulfonylurea drugs.

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In a recent study Mirsky, *et al.* (14), observed that administration of tolbutamide to dogs who received growth hormone diminished or prevented the usual hyperglycemic response to the hormone. These findings seem to suggest that in this instance adequate pancreatic reserve existed despite the administration of growth hormone. The inference could also be drawn that B cell destruction after growth hormone is not due to excessive stimulation of B cells since the sulfonylureas, which act directly upon them, did not potentiate the diabetogenic effect of the growth hormone. However, these authors did not study the pancreas histologically. It, therefore, seemed of interest to investigate the diabetogenicity of growth hormone in dogs whose pancreatic B cells had been stimulated by pretreatment with tolbutamide and in whom the insulinogenic reserve had been reduced by 50% partial pancreatectomy. Furthermore, the diabetogenic effectiveness of cortisone was investigated in rabbits each of which received tolbutamide prior to and during hormonal administration.

MATERIAL AND METHODS

A) *Dogs*: Thirty-eight mongrel dogs of either sex, weighing from 12 to 15 kg. were 50% partially pancreatectomized. On or after the 14th post-operative day 17 animals received 0.5 gm. b.i.d. of tolbutamide or of metahexamide by mouth for periods of 2 to 83 days. Eleven were given a similar dose of the sulfonylurea as well as 3 mg./kg. of purified growth hormone[2] intramuscularly daily for 5 to 21 days. Ten dogs received similar dosages of growth hormone alone for periods of 3 to 22 days. All dogs were fed a high carbohydrate diet consisting of mashed potatoes, meat and meat gravy and 5% glucose solution *ad libitum* instead of drinking water. Morning fasting specimens for glucose determination were withdrawn daily from the leg veins.

B) *Rabbits*: Twenty-four rabbits of either sex, weighing from 2500 to 4000 gm. received 125 mg./kg. of tolbutamide or metahexamide intravenously for periods of up to 35 days. Starting on the 14th day seven of these animals also were given 4 mg./kg. of cortisone acetate[3] for up to 14 days. An additional 12 rabbits received a similar dosage of cortisone alone daily for 23 days and served as controls. All animals were kept in individual metabolic cages and received Purina rabbit chow and water *ad libitum*. Each animal received 200,000 units of procaine penicillin G and 0.25 gm. of dihydrostreptomycin sulfate (Combiotic-Pfizer) twice weekly. Blood was drawn from the marginal ear vein daily in the morning at approximately 9:30 A.M. and in the afternoon at approximately 1:30 P.M.

All surviving rabbits and dogs were sacrificed by overdosage with nembutal on the morning following the last dose of medication and portions of the pancreas were placed in Zenker-Formol solution. Blocks were processed as described previously and sections were stained by a modification of the Masson trichrome stain (15), the aldehyde fuchsin method (16) and the PAS trichrome technique controlled by diastase digestion for glycogen identification (17). For demonstration of fat other blocks of tissue were embedded in Carbowax and stained with Sudan IV. The blood sugar determinations were carried out by the Nelson-Somogyi method (18).

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[2] Purified growth hormone was a gift from the Endocrine Study Section of the National Institute of Health.

[3] Cortisone acetate was supplied by the Upjohn Company, Kalamazoo, Michigan, through the courtesy of Dr. C. J. O'Donovan.

RESULTS

Blood sugars

Dogs: The morning blood sugar values in untreated partially depancreatized dogs varied from 84 to 125 mg.%. In similar animals who received sulfonylureas the values ranged between 67 and 125 mg.%. These levels are not significantly different from those of the normal. Of the 10 dogs which received growth hormone alone 8 developed diabetes between the 2nd and 5th day after the start of hormonal treatment. At the time of sacrifice the blood sugar levels of these 8 animals varied between 225 and 415 mg.%. In 9 of the 11 dogs who received sulfa drugs as well as growth hormone, a marked hyperglycemia began on the 2nd to the 6th day after starting hormonal treatment. This hyperglycemia persisted until sacrifice of the animals. The terminal blood sugar levels of these animals varied from 191 to 435 mg.% (Fig. 1).

Rabbits: The daily morning blood sugars of the sulfonylurea treated rab-

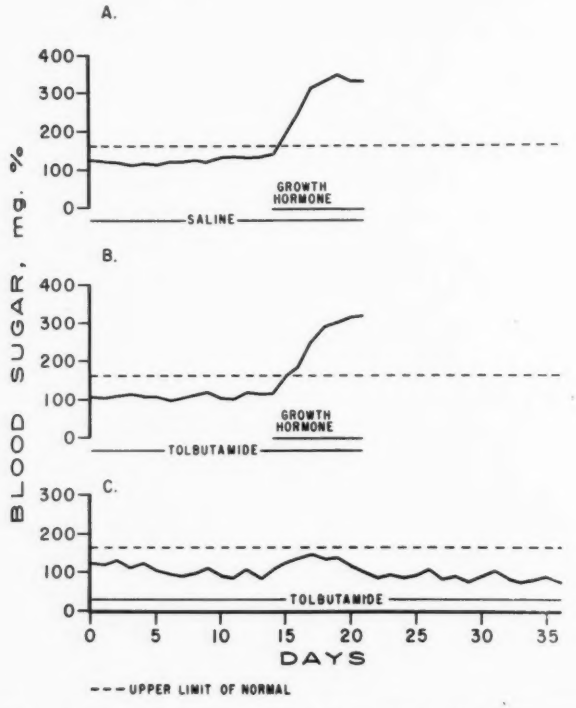


FIG. 1: Daily morning blood sugar levels of 3 representative 50% partially depancreatized dogs which received respectively: A) Saline solution and later also 3 mg./kg. orally of growth hormone daily s.c., B) 0.5 mg. b.i.d. of tolbutamide orally and later 3 mg./kg. of crystalline growth hormone s.c. in addition. C) Only 0.5 mg. b.i.d. of tolbutamide orally.

bits were usually within normal values and ranged from 72 to 122 mg.%. The afternoon blood sugar concentration was frequently considerably decreased ranging down to 65 mg.%. Rabbits who received cortisone treatment alone showed a marked rise of the blood sugar by the 4th to 6th day which reached its peak of 242 to 416 mg.% on the 10th to 14th day. From then on there was usually a gradual decline of the hyperglycemic level. Animals which were treated with the sulfonylurea drugs in addition to cortisone showed blood sugar curves similar to those observed in the rabbits which received steroid treatment alone.

Pancreatic morphology

Dogs: The untreated partially pancreatectomized dogs revealed no significant pancreatic changes. Of the sulfonylurea-treated animals 3 showed partial and 14 showed almost complete B cell degranulation. Complete degranulation usually became apparent at the 7th to 8th day after the start of treatment. The B cells showed increased size of the nuclei as well as an unusual prominence of the negative image of the Golgi apparatus (cf. Fig. 2a and 2b). There was no evidence of any vacuolization or destruction of the B cells.

Animals treated with growth hormone alone or those who received both the hormone and sulfonylurea displayed similar morphologic changes in the pancreas. Concomitant with the development of the hyperglycemia there was increase in glycogen infiltration in ductular epithelium as well as in an occasional acinar cell. The B cells in addition to progressive degranulation, showed two types of vacuolization. One type consisted of single or multiple cytoplasmic glycogen-containing vacuoles. The other consisted of ballooned-out cells which progressively increased in numbers. These cells contained pyknotic nuclei which were connected to the cell membrane by thin strands of cytoplasm (Fig. 3a and 3b). The vacuoles of these cells contained neither glycogen nor fat. Eventually there was a marked decrease of the number of islets. These residual islets consisted only of A cells and D cells as well as of not clearly identifiable cells which are probably of ductular origin.

Animals treated with growth hormone alone or together with sulfonylureas which did not develop diabetes showed the expected small amounts of glycogen in pancreatic ductular epithelium. The B cells were not vacuolated and did not contain glycogen but showed frequently extensive degranulation. The A cells and D cells were unchanged.

Rabbits: The rabbits treated with sulfonylurea alone showed varying degrees of B cell degranulation which commenced on the 3rd and frequently was complete by the 7th or 8th day (compare Fig. 4a and 4b). In addition there was marked enlargement of the nuclei as well as of the negative image of the Golgi apparatus. Those animals which were treated with both sulfa drugs and cortisone showed B cell degranulation as well as glycogeni-

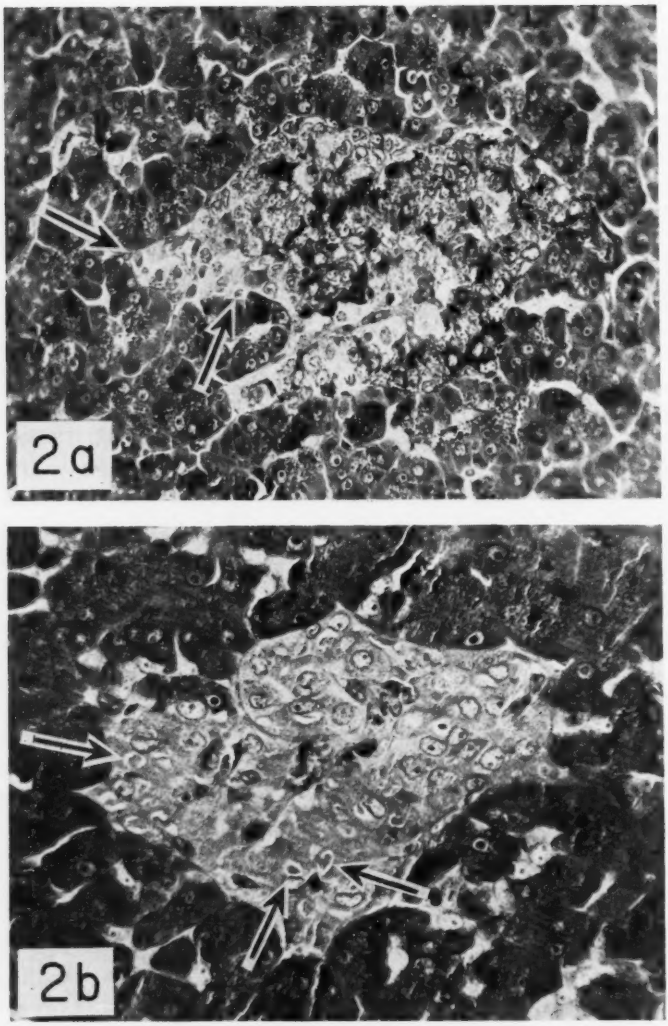
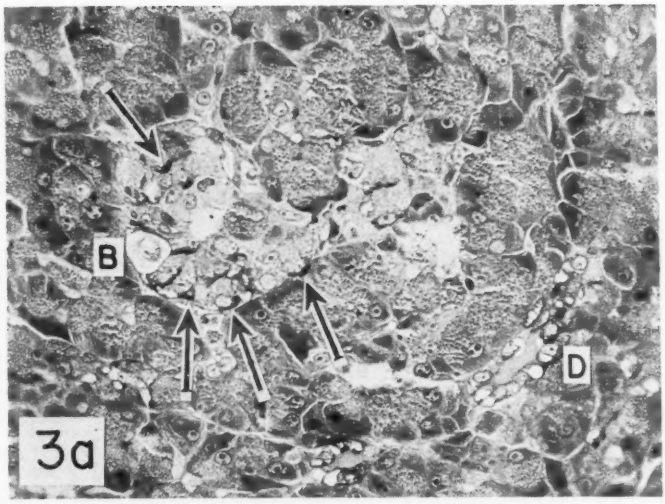


FIG. 2a: Pancreatic islet of normal dog showing well granulated B cells. The A cells are pale (arrows). Aldehyde-Fuchsin-Trichrome. ×375.

FIG. 2b. Pancreas of 50% partially pancreatectomized dog treated with 0.5 gm. of metahexamide daily orally for 52 days. There is almost complete degranulation of B cel's. Moreover, there is enlargement of B cell nuclei and Golgi apparatus (arrows). P.A.S. Trichrome. ×500.



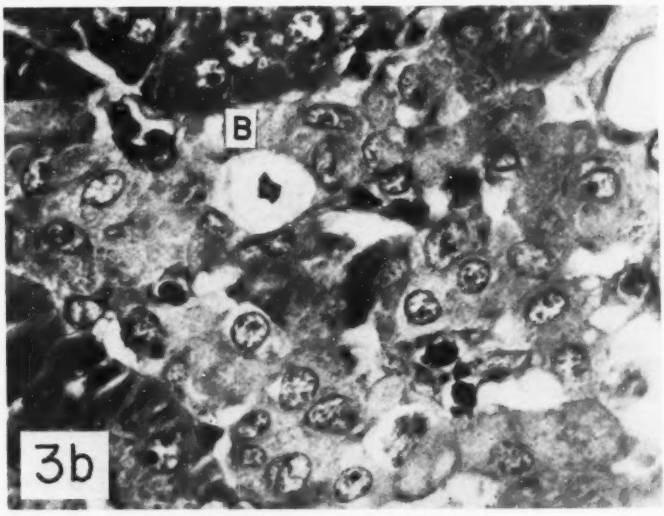


FIG. 3a. Pancreas of 50% partially pancreatectomized dog treated with 0.5 gm. of tolbutamide b.i.d. orally and later also with 3 mg./kg. of growth hormone s.c. There is glycogenization of ductular epithelium (D) and B cells (arrows) as well as ballooning vacuolization of a B cell (B). P.A.S. Trichrome. ×960.

FIG. 3b. High power view of islet of 50% partially pancreatectomized dog treated with 0.5 gm. of tolbutamide b.i.d. orally and later also with 3 mg./kg. of growth hormone. The B cells are extensively degranualted. There is a ballooned-out vacuolated B cell with pyknotic nucleus present (B). Aldehyde-Fuchsin-Trichrome. ×375.

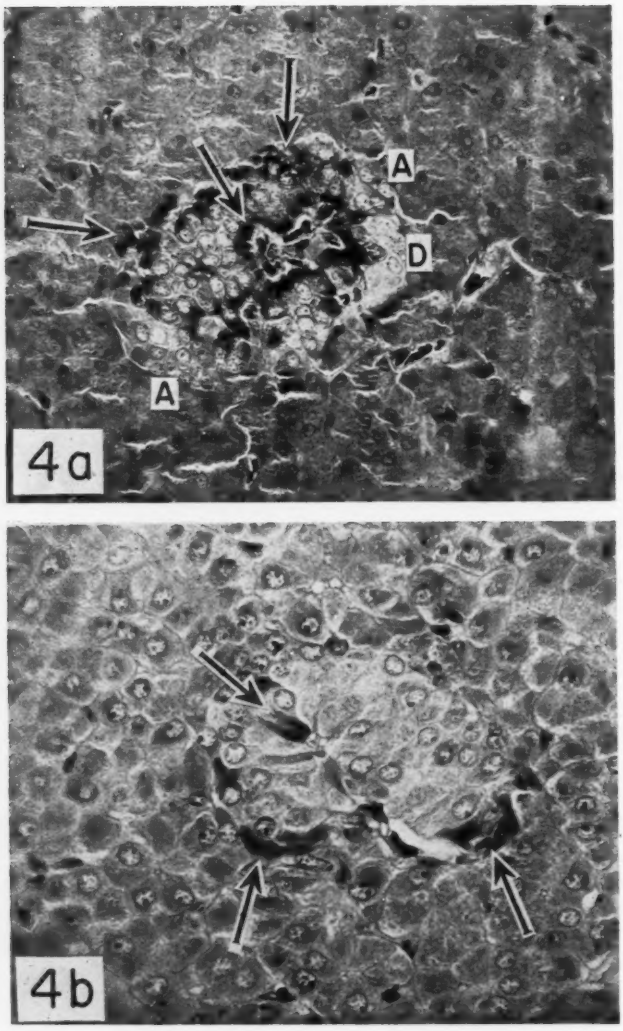


FIG. 4a. Pancreas of untreated rabbit showing A and B cells. The B cell granules are polarized around the capillary borders of the cells (arrows). The A cells are mediun gray (A), the D cells are light gray (D). Aldehyde-Fuchsin-Trichrome. ×375.

FIG. 4b. Pancreas of rabbit treated for 27 days with 125 mg./kg. of metahexamide i.v. There is complete degranulation of B cells but no other changes. The A cells are dark (arrows). Aldehyde-Fuchsin-Trichrome. ×500.

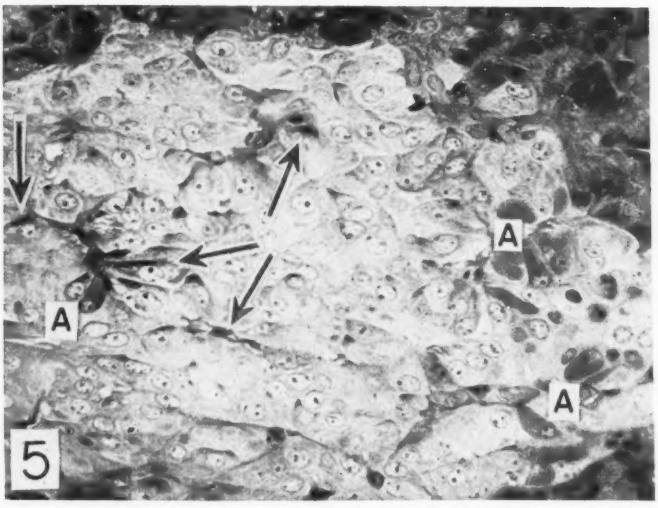


FIG. 5. Pancreas of moderately diabetic rabbit treated for 19 days with 125 mg./kg. of metahexamide as well as 4 mg./kg. of cortisone acetate daily. The B cell nuclei are somewhat enlarged and there is mild glycogenization (arrows). No degenerative changes in B cells are seen. The A cells are black (A). P.A.S. Trichrome. ×375.

zation of B cells and duct epithelium commensurate with the degree of diabetes which developed. There was, in addition, enlargement of B cell nuclei and Golgi apparatus and some apparent increase in size of islets. However, there was no evidence of any destruction of B cells (Fig. 5). Animals treated with cortisone alone showed similar morphologic alterations.

DISCUSSION

The results of the present study which seem to be at variance with those of Mirsky, *et al.* (14) demonstrate that in the 50% partially pancreatectomized dog, hypoglycemic sulfonylureas, in the majority of animals, did not prevent the diabetogenic response to growth hormone. However, in the present study the pancreatic reserve had been diminished by prior surgery whereas in the previous study intact dogs were utilized. Furthermore sulfonylurea administration in rabbits did not particularly alter the diabetogenic response to cortisone. These findings support our previous observation that tolbutamide will not cause hypoglycemia in growth hormone treated dogs or cortisone treated rabbits unless some pancreatic reserve is present (13).

The B cell destruction in dogs during hormone administration has been shown to be due to progressive development of necrobiotic B cells with ballooning vacuolization of cytoplasm and nuclear pyknosis (19). This lesion differs from vacuolization due to glycogen infiltration which is also found

in dogs and is the only type seen in the rabbit. This latter form of vacuolization is a reversible metabolic process reflecting the elevated concentration of glucose in the tissue fluids and is not a destructive lesion (19–21).

The hypothesis that vacuolization and destruction of B cells during diabetes in dogs represents overstrain of these cells due to increased demands for insulin was originally put forward by Allen (23, 24) and Homans (25, 26) and since has been generally accepted. This mechanism is used to explain the pathogenesis of the permanent diabetes produced by various diabetogenic agents in the dog and cat. That prolonged blood sugar elevation is responsible for B cell destruction seems to have been proved by the fact that in cats prevention of hyperglycemia prevented B cell destruction (6), whereas massive doses of glucose have produced it (27, 28). This was substantiated in the present study by the fact that in the two dogs who did not become diabetic when treated with growth hormone following tolbutamide no ballooning vacuolization of B cells was seen. The mechanism whereby hyperglycemia causes B cell destruction in the dog is, however, not known. It seems unlikely that this is due to a functional exhaustion of the B cells. If it were, it would be remarkable, since, as a general rule, excessive stimulation results in cell hypertrophy and hyperplasia rather than destruction. Moreover, if functional exhaustion were the mechanism one would expect the sulfonylureas, which also stimulate B cells, to reinforce the B cell destructive action of growth hormone induced diabetes. Also in no instance was chronic sulfa drug administration associated with vacuolization or destruction of B cells despite morphologic changes in the pancreas including degranulation and enlargement of Golgi apparatus which are those of chronic B cell stimulation.

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VASOPRESSIN IN BLOOD: EFFECT OF HEMORRHAGE[1]

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ABSTRACT

A method has been developed for the estimation of vasopressin in blood. By means of a few simple chemical and chromatographic procedures vasopressin contained in large quantities of blood may be extracted and obtained in small volumes of solution suitable for either bioassay determinations or further purification. These methods coupled with a bioassay procedure have been employed for the study of the time course of changes in the concentration of pressor material in the blood after hemorrhage. Evidence is presented that this pressor material is identical with vasopressin. From the results of these studies, an approximate rate and total discharge of vasopressin into blood of dogs subjected to hemorrhage was calculated.

THE concentration of vasopressin (antidiuretic hormone, ADH) in the blood of various mammals has been determined by antidiuretic assays of either whole blood, plasma or serum (cf. 1, 2, 3). The antidiuretic response induced by the injection of blood plasma or serum into assay animals has often been attributed to antidiuretic hormone (ADH). However, the antidiuretic assay is not specific enough to justify the conclusion that the response of the test animal is due either solely or in large measure to vasopressin in these body fluids (cf. 1, 2).

In this paper we wish to report on the development of simple chemical and chromatographic procedures wherein minute quantities of vasopressin contained in large volumes of blood may be extracted, purified and concentrated. The coupling of these chemical and chromatographic steps with a bioassay procedure confers considerable specificity on the quantitative estimation of vasopressin in blood. Such methods have now been applied to the determination of the rate of discharge of vasopressin into the blood of dogs which have been subjected to hemorrhage and maintained at a blood pressure of 50 mm. of mercury.

MATERIALS AND METHODS

Mongrel dogs were used as a source of blood and as experimental animals. Pressor assays were performed on the rat by the method described in Pharmacopoeia of the

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[2] Postdoctoral Research Fellow of the National Institute of Arthritis and Metabolic Diseases, United States Public Health Service.

United States (1955). The U.S.P. standard had a stated potency of 0.47 Units per mg. The mean potency estimates of unknown samples are expressed in terms of mU. of vasopressin; numbers in parentheses represent 95% fiducial limits.

Recovery of Vasopressin Added to Blood. In the first series of experiments, dogs were anesthetized by intravenous injection of a 3% sodium pentobarbital solution (1 cc. per kg.), heparinized, and bled rapidly from a femoral artery. Known quantities of vasopressin (U.S.P. posterior pituitary standard dissolved in 0.9% sodium chloride) were mixed with 100 cc. aliquots of this blood (previously cooled in ice), and used for recovery experiments. Control samples of blood to which no vasopressin was added were run simultaneously.

In a second series of experiments, vasopressin was introduced into the lumen of a cannula emerging from the femoral artery. The site of injection was just proximal to a screw clamp which regulated blood flow during collection to 50 to 100 cc. per minute. The endogenous source of vasopressin had been removed prior to collection of the samples.[3]

One hundred cc. aliquots of whole blood collected by either of the two procedures described above and containing known quantities of vasopressin, were added to or collected in 200 cc. of cold 15% trichloroacetic acid (TCA), immersed in an ice bath and magnetically stirred. After stirring for about 10 minutes, the suspension was centrifuged; the precipitate was washed successively with 100 cc. of 10% TCA and twice with 50 cc. portions of 5% TCA. The combined washings and supernatant solution were filtered, and extracted three times with an equal volume of ether. The clear aqueous solution was adjusted to pH 4.5 (glass electrode) with dilute NH_4OH and passed through a column of XE-64 (H form in H_2O) 2 cm. in diameter and 1 cm. high at a flow rate of about 2 cc. per minute. The XE-64 (H form) resin had been prepared as described by Hirs *et al.* (4), and then washed successively with a 30% pyridine-4%-acetic acid solution, H_2O, 2 N HCl and H_2O. After the blood extract had passed through the column, the resin was washed with 20 cc. of H_2O and vasopressin was subsequently eluted with 25 cc. of 30% pyridine-4%-acetic acid (5). The eluate was collected in a vessel containing 3 mg. of L-cystine (California Foundation) dissolved in 0.5 cc. of 0.1 N HCl. All column operations were carried out at 4° C. The eluate was concentrated to a syrup *in vacuo* at a temperature below 30° C. The remaining traces of pyridine were removed by the repeated addition of H_2O (3 to 4 times) and subsequent concentration *in vacuo*. The residue was suspended in a known volume of sodium chloride (0.15 M)-acetic acid (5×10^{-3} M) and centrifuged; the supernatant was assayed for pressor activity.

Extraction and Assay of Endogenously Secreted Vasopressin Before and After Hemorrhage. In the first series of experiments (Procedure A) blood was collected before and after hemorrhage from a tributary of the external jugular vein at its point of emergence from the jugular foramen. It was anticipated that blood obtained from this vein would have a relatively high concentration of vasopressin. In the second series of experiments, Procedure B, was employed; blood samples were collected from the superior vena cava in an animal in which the forelegs, neck and lower jaw were excluded from the circulation. The details of the two procedures will now be presented.

In Procedure A, the dog was anesthetized with pentobarbital (30 mg. per kg. I.V.) and given heparin. Cannulae were inserted into a femoral vein, both femoral arteries, a common carotid artery, and a branch of the external jugular vein which drained the region of the cavernous sinus. The cannula in the femoral vein was used for infusion of

[3] The dogs were anesthetized with barbiturate and heparinized. Artificial respiration was instituted; the chest was opened and the left subclavian and brachiocephalic arteries ligated. The calvarium was removed and the brain (down to spinal cord and including pituitary) lifted out.

either saline, fresh whole blood from donor dogs, heparin or additional anesthetic during the course of the experiment. The tubes leading from the arterial cannulae were clamped until it was desired to lower the blood pressure to 50 mm. Hg. Just prior to opening the clamps (hemorrhage), 100 cc. of blood (control sample) was collected from the aforementioned branch of the jugular vein; simultaneously 100 cc. of donor blood was infused via the femoral vein. The arterial clamps were then opened (zero time) and within 30 seconds the blood pressure reached a value of about 50 mm. of mercury during which time 400 to 800 cc. of blood entered a pressure bottle. The pressure was maintained at 50 mm. of mercury for the duration of the experiment. At various time intervals, 100 cc. samples of venous blood (jugular branch) were collected directly into 200 cc. of ice cold 15% TCA (magnetically stirred). Arterial blood samples were usually collected simultaneously and processed as described for the venous samples. Vasopressin was recovered and assayed.

In Procedure B, the dog was anesthetized and given heparin; the femoral vein and arteries were cannulated as described above. A thoracotomy was performed and the animal supported with artificial respiration. The brachiocephalic artery was cannulated at its origin and connected by a Y tube and polyethylene tubing to both internal carotid arteries. The left subclavian artery was ligated close to the aorta and the brachiocephalic artery just distal to the point of cannulation. These manipulations bypassed the circulation to the forelegs, neck and lower jaw. The internal mammary and subclavian veins were occluded. An "L" shaped cannula was inserted into the superior vena cava via the azygos vein. By means of a "choke" the superior vena cava could be tightly compressed around the cannula, thereby directing blood from the dog's head through the cannula. Between collections of venous blood from the head, blood flowed around the cannula into the right atrium. At the start, the "choke" was applied to the superior vena cava so that blood draining the head was diverted through the "L" cannula emerging from the azygos vein. Immediately after the control sample was collected, the clamps on the femoral arterial cannulae were released in order to lower the blood pressure to 50 mm. Hg. Thirty seconds after the clamps had been released, three 100 cc. venous samples were collected at thirty second intervals. Thereafter, the "choke" was released and subsequently applied during the collection of venous samples taken after more extended periods. Arterial blood samples were collected from a femoral artery simultaneously and at a rate regulated to coincide with the collection of venous blood. The time required to collect each 100 cc. sample of blood was measured with a stopwatch.

RESULTS

Recovery of vasopressin added to blood. The mean recovery value for arginine vasopressin added in the form of U.S.P. Posterior Pituitary Reference Standard (0.47 U/mg.) to whole blood was 59.8 per cent with a standard error of ± 1.46 (Table 1). The per cent of vasopressin recovered was essentially independent of the initial concentration over the range studied (50 to 400 mU. per 100 cc. of blood). In six experiments, partially purified arginine vasopressin (65 U/mg., Wilson lot No. 110–127500) was used, and similar recovery values were obtained. In order to determine whether blood components interfered with the pressor bioassay, "blank" blood samples were carried through the recovery procedure. The potency estimates of known quantities of vasopressin dissolved in the final, concentrated column eluates (which in most instances contained negligible or immeasurable amounts of pressor material) were not different from the

TABLE 1. RECOVERY OF VASOPRESSIN ADDED TO DOG BLOOD

No. determinations	mU. per 100 cc. of blood		Per cent recovered
	Added	Recovered	
5	50	26 ± 2.5*	52 ± 5.0*
11	80	53 ± 1.2	67 ± 1.5
6	100	57 ± 3.9	57 ± 3.9
3	160	95 ± 9.7	59 ± 4.1
7	200	117 ± 14.9	58 ± 2.8
4	400	227 ± 21.0	57 ± 1.7
Mean recovery = 59.8 ± 1.46%**			

* Standard error calculated for the mean of the potency estimates of individual assays.
** Mean ± standard error calculated for the mean of the potency estimates of individual assays for 36 recovery experiments.

potency of a saline solution containing the same amount of vasopressin.

Effect of Hemorrhage on the Concentration of Vasopressin in Blood. In Table 2, are presented the concentrations of pressor material in blood obtained from a branch of the jugular vein which drained the cavernous sinus, before and after hemorrhage of the dog to a blood pressure of approximately 50 mm. Hg (Procedure A). The quantity of pressor material in the pre-hemorrhage samples was either not detectable or very small. However, after lowering the blood pressure to 50 mm. Hg, enough vasopressin could be recovered (80 to 497 mU./100 cc. blood) for precise bioassay measurements. The concentration given (corrected for 60% recovery) for each experiment represents the maximum value obtained for a series of blood samples collected over the period (up to 5 hours) during which the blood pressure was maintained at 50 mm. Hg. Approximations of the pressor concentrations in arterial blood collected over the same time interval are also recorded. It is apparent from inspection of Table 2 that considerable quantities of pressor material are present in jugular vein blood after the blood pressure has been lowered to 50 mm. Hg. The concentration of pressor activity was high enough in these samples to warrant the application of

TABLE 2. CONCENTRATIONS OF VASOPRESSIN IN BLOOD OF DOGS BEFORE AND AFTER HEMORRHAGE

Experiment No.	Milliunits Vasopressin per 100 cc. of Blood*		
	Jugular Vein**		Carotid Artery
	Zero Time	Post Hemorrhage#	Post Hemorrhage
1	<10	80 (71 to 90)	47
2	—	497 (463 to 533)	95
3	<10	481 (458 to 503)	42 (37 to 48)

* All values have been corrected for a recovery of 60%; numbers in parentheses represent 95% fiducial limits.
** A branch which drained the cavernous sinus.
# In Exp. No. 1, 100 cc. of blood was collected over the 40th to 50th minute post hemorrhage and was the first sample taken; Exp. No. 2, collection started 1 minute after opening the arterial clamps; first 50 ml. collected over a period of approximately 5 minutes; last 50 ml. of blood requiring 55 minutes more to collect; Exp. No. 3, started 5 minutes post hemorrhage, collected over a period of approximately 5 minutes.

additional steps for the purification and identification of this pressor material.

Identification of vasopressin in blood. The biological, chemical and chromatographic properties of the pressor material found in the blood of dogs subjected to hemorrhage support the conclusion that this substance is vasopressin. The biological evidence includes the observations that the head is the source of the material and that it is capable of eliciting a vasopressor response in the phenoxybenzamine-treated rat. In addition, the pressor material found in blood after hemorrhage showed chemical and chromatographic properties which were (by all criteria employed) identical with vasopressin. For example, this material was soluble in 10% TCA, insoluble in ether, stable to the action of pepsin, rapidly inactivated by trypsin and by 0.01 M thioglycollic acid (pH 7.4), and readily adsorbed onto and eluted from a cation exchange resin (XE–64) under conditions identical with those observed with authentic samples of vasopressin.

In a separate experiment, 500 cc. of blood was collected continuously over a period of 19 minutes from a branch of the jugular vein (Procedure A) beginning 30 seconds after the blood pressure had been lowered to 50 mm. Hg. The total amount of vasopressor material recovered was 712 (653 to 780) mU. When an aliquot of this material, 640 (587 to 702) mU., was adsorbed onto carboxymethyl cellulose (0.9×10 cm., 0.02 M ammonium acetate, pH 6.0) and eluted by a continuous gradient elution procedure (6), 292 (272 to 318) mU. of pressor material emerged as a single sharp peak in approximately the same position previously observed with either highly purified vasopressin or crude hypophyseal extracts (7, 8).

Time course and rate of discharge of vasopressin into blood after hemorrhage. In order to obtain some measure of the rate of discharge of vasopressin and of the total amount of this hormone appearing in the blood after lowering the blood pressure to 50 mm. Hg, Procedure B was adopted. In these experiments, practically all blood draining the head was collected and rate of flow measured. The time course of changes in the concentration of vasopressin in the blood obtained from the superior vena cava after hemorrhage is shown in Figure 1. Approximations of arterial blood samples are also given. During the first few minutes following hemorrhage there is a massive discharge of vasopressin into the blood. Since the time required to collect each 100 cc. sample was also determined, an approximate rate (mU./min.) may be calculated. For the two experiments, the highest rate of discharge of vasopressin observed coincided with the peaks of activity shown in Figure 1, and was estimated to be approximately 142 and 297 mU./min., respectively. In a third experiment not shown, the massive discharge of vasopressin following hemorrhage was not observed. However, this dog was obviously in poor condition as indicated by the fact that the blood pressure dropped to 50 mm. Hg after only 100 cc. of blood entered the pressure bottle.

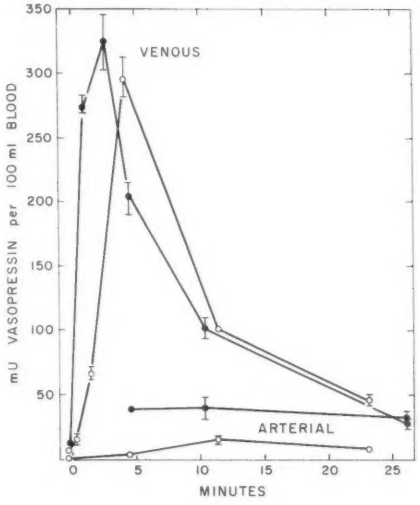

FIG. 1. Time course of discharge of vasopressin into the blood of dogs which have been rapidly bled and maintained at a blood pressure of 50 mm. Hg. ○———○, dog No. 1; ●———●, dog No. 2.

Experiment performed as described in the text (Procedure B); venous blood obtained from the superior vena cava; arterial blood from a femoral artery. Bleeding initiated at zero time; vertical bars represent 95 per cent fiducial limits of potency estimate; all values have been corrected for a recovery of 60 per cent.

Points are plotted at the middle of the interval required to collect each 100 cc. blood sample. These collection times in minutes were for dog No. 1: control sample, 0.36; post hemorrhage, 0.36, 1.0, 2.10, 2.4, and 1.38; for dog No. 2: control sample, 0.23; post hemorrhage, 0.93, 1.33, 1.1, 0.88, and 0.58.

DISCUSSION

The procedure for the recovery of vasopressin in blood described in this report represents modifications of techniques devised for the isolation of labeled vasopressin from the hypothalamus and the neurohypophysis of the dog (7, 8). The mean recovery of vasopressin added to blood was approximately 60 per cent and appeared to be independent of the initial concentration (50 to 400 mU./100 cc. blood). An important advantage of the extraction procedure is the considerable specificity which is conferred as a result of the nature of the chemical and chromatographic steps involved. The sensitivity of the pressor assay employed determined the lower limits of concentration of hormone that could be estimated. Sensitive antidiuretic assays (cf. 2, 3) may extend the application of the procedure to relatively low concentrations if the extraction procedure recovers approximately the same percentage of hormone as described above. Furthermore, the extraction procedure may in all likelihood be applied to the determination of oxytocin in blood.

Relatively large quantities of vasopressor material appeared in the effluent blood from the cavernous sinus in response to the stimulus of lowering the blood pressure to 50 mm. Hg. These results confirm reports from a number of laboratories dealing with the discharge of antidiuretic substance into the blood after hemorrhage (3, 9, 10). However, the conclusions reached in these reports must be qualified because of the failure to demonstrate the specificity of the methods employed. The biological, chemical, enzymatic and chromatographic data obtained in the present

study offer convincing evidence that the vasopressor material that we are dealing with is indeed the hormone, vasopressin.

The results of our initial experiments wherein blood was collected from a branch of the jugular vein suggested that the concentration of vasopressin in the blood reached its peak activity early after the blood pressure was lowered to 50 mm. Hg (cf. Reference 10). These results were confirmed by extraction and assay of blood samples collected from the superior vena cava. The massive discharge of vasopressin within a few minutes after lowering the blood pressure was striking (Figure 1). Since the time required to collect each 100 cc. sample was also determined (See Legend, Figure 1), an approximate rate (mU./min.) may be calculated. For the two experiments, the area under the curve for a plot of mU./min. versus time (corrected for the arterial concentrations) gave 1,270 and 1,900 tota mU., respectively, discharged over a period of twenty minutes. The magnitude of this outpouring of vasopressin into blood becomes all the more significant in view of the reasonable assumption that these numbers represent minimal values (not taken into account is the drainage of blood from the head via pathways other than those leading into the superior vena cava).

If the observed massive discharge of vasopressin into blood reflects the synthetic activity of the hypothalamo-neurohypophyseal system then the rate of synthesis of vasopressin after hemorrhage would be several orders of magnitude greater than the estimated resting value (11). This question may be investigated by the application of tracer methods used for the study of the incorporation of S^{35} labeled cystine into vasopressin (7, 8).

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WORK PERFORMANCE OF A RAT HEART-LUNG PREPARATION: STANDARDIZATION AND INFLUENCE OF CORTICOSTEROIDS[1,2]

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ABSTRACT

Experimental conditions have been established for the operation of a rat heart-lung preparation which result in a high degree of uniformity of work performance as measured by left ventricular work index (LVWI) (proportional to the product of cardiac output and mean arterial blood pressure). At a perfusion blood temperature of 37° C heart rate was higher and left ventricular work index lower than at 32° C. Peripheral resistance was adjusted to give an analysis of the work performance of the heart-lung at three different levels of mean arterial blood pressure: 80, 110, and 130 mm. Hg. The lower the pressure, the greater LVWI. Heart rate was not influenced by alterations in peripheral resistance.

LVWI was less in heart-lung preparations perfused with blood from adrenalectomized rats than in preparations perfused with blood from intact rats. Corticosterone, cortisol or aldosterone was added to perfusion blood from adrenalectomized rats to yield a concentration of 10 μg., 10 μg. and 3.2×10^{-2} μg. per 100 ml., respectively. These corticosteroids increased LVWI. On the other hand, neither Substance S nor tetrahydrocortisol at 10 μg. per 100 ml. had an effect on LVWI.

A wide range of concentrations (2, 10, 50, and 500 μg. per 100 ml.) of corticosterone and of tetrahydrocortisol was tested in the rat heart-lung at a perfusion blood temperature of 32° C and of 37° C. Tetrahydrocortisol did not increase LVWI at any concentration tested. At high concentrations, this steroid decreased LVWI; the inhibitory concentration at 32° C was less than the inhibitory concentration at 37° C.

Corticosterone was effective in increasing LVWI at a concentration of 2 μg. per 100 ml. at 32° C, but was ineffective at this concentration at 37° C. At the higher temperature, corticosterone exerted a stimulatory action in a concentration of 10 μg. per 100 ml. High concentrations of corticosterone were inhibitory, and as in the case of tetrahydrocortisol, the inhibitory concentration was less for the heart-lung operating at 32° C than at 37° C. These observations indicate that the stimulatory and inhibitory concentrations of corticosterone in the rat heart-lung are temperature dependent.

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[3] Arthritis and Rheumatism Foundation Fellow, 1956–present.

IN A previous communication from this laboratory (1) a rat heart-lung preparation has been described. The preparation was characterized by relatively high reproducibility of work performance and by responsiveness to the titer of corticosteroids in the perfusion blood. Additional quantitative studies have yielded more precise information as to factors such as peripheral resistance and temperature of perfusion blood which influence the work performance of this isolated system. The corticosteroids which exert a stimulatory action on the heart-lung have now been demonstrated to include corticosterone, cortisol, and aldosterone.

METHODS

The essential features of the isolated rat heart-lung are illustrated in Figure 1. The pulmonary vasculature was in the circuit, but the lungs were not ventilated and re-

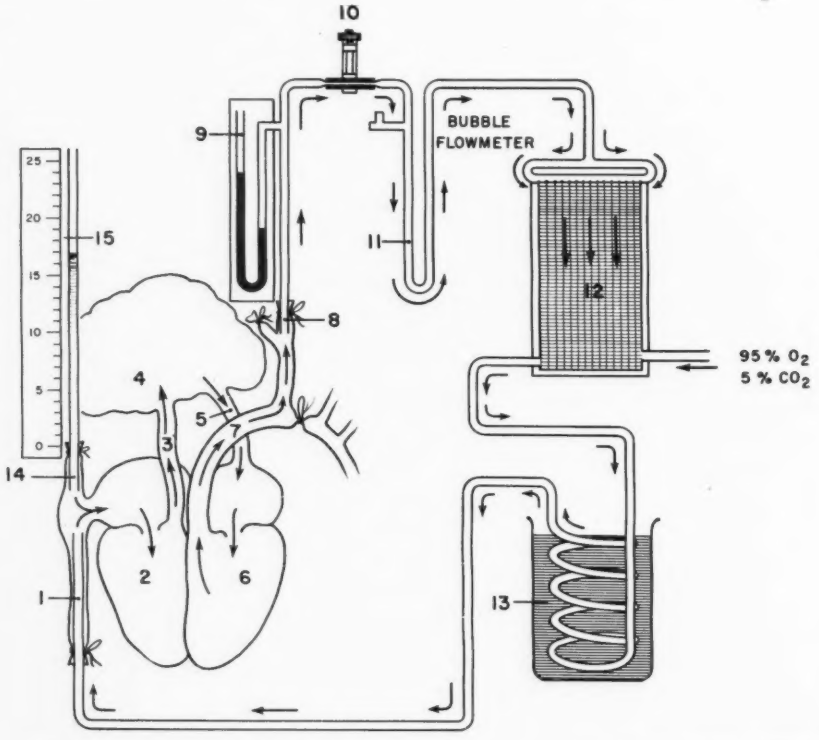

FIG. 1. Diagrammatic scheme of rat heart-lung preparation. Arrows show direction of blood flow. Numbers correspond to the following: 1, Cannulated Inferior Vena Cava; 2, Right Ventricle; 3, Pulmonary Artery; 4, Lung; 5, Pulmonary Vein; 6, Left Ventricle; 7, Aorta; 8, Cannulated Right Common Carotid Artery; 9, Mercury Manometer; 10, Peripheral Resistance Clamp; 11, Bubble Flow Meter; 12, Screen Oxygenator; 13, Constant Temperature Water Bath; 14, Cannulated Superior Vena Cava; 15, Saline Manometer.

mained collapsed during the course of an experiment. The blood was oxygenated on a screen oxygenator.

Mean arterial pressure was maintained at a preselected level during the course of an experiment by adjustment of the peripheral resistance clamp (Fig. 1). Cardiac output was measured at frequent intervals until the preparation could no longer expel blood at the preselected level of arterial pressure.[4] The measure of work performance, left ventricular work index (LVWI), is proportional to the product of the sum of the cardiac outputs of successive intervals and the mean arterial blood pressure (MABP). $LVWI = \Sigma(C\ O_t)(MABP)(K)$, in which $C\ O_t$ is the cardiac output for a given interval t; $\Sigma(C\ O_t)$ is the sum of the cardiac outputs in ml. for the successive intervals; MABP is expressed in mm. Hg. (constant for a given experiment); K, a conversion factor equal to 0.0136, is used to express LVWI as gram-Meters of work. (See Legend to Fig. 2 for calculation of LVWI). LVWI does not include that fraction of left ventricular work required to perfuse the coronary arteries or that fraction of left ventricular work required to accelerate the blood in its exit from the left ventricle. For these reasons the evaluation of the performance of the left ventricle calculated by the above equation is designated an *index*.

Average heart rate was calculated from ECG recordings taken at 30 minute intervals. Right atrial pressure[5] was recorded by means of a saline manometer connected to a cannula in the superior vena cava; the tip of the cannula was at the level of the right atrium.

Albino male rats weighing 320–370 grams were obtained from the Holtzman Farms and acclimatized for at least one week prior to their use as blood donors or as animals from which heart-lungs were prepared. Bilateral adrenalectomy was performed two to three weeks before the isolation of the heart-lung; certain animals were subjected to sham adrenalectomy. The anesthetic for all surgical procedures was sodium pentobarbital, 40 mg./Kg., administered intraperitoneally as a one percent aqueous solution. Adrenalectomized animals were maintained post-operatively on 0.9% sodium chloride solution; sham adrenalectomized animals drank tap water. Both groups were housed in a constant temperature room for at least two weeks before isolation of the heart-lung. They were fed Purina laboratory chow *ad libitum*. Completeness of adrenalectomy was checked by inspection of the suprarenal area.

Perfusion blood was withdrawn from the abdominal aortae of heparinized donor rats. Ethanol solutions of the steroids were pipetted into siliconized 100 ml. beakers and dried under vacuum. The perfusion blood was then added to the dried steroid, stirred gently, and transferred to the heart-lung. The tubing, the screen oxygenator, the stopcocks, the hypodermic needles and all glassware were siliconized.

The influences of seasonal variation and endemic infections were minimized by arranging the experiments in a rotation. For example, in an experiment designed to determine the influence of temperature on LVWI the following sequence was replicated until there were 12 preparations in each group: Intact-Intact (heart-lung from intact rat perfused with blood from intact rats) at a perfusion blood temperature of 32° C; Intact-Intact at 37° C; Intact-Adrenalectomized (heart-lung from intact rat perfused with blood from adrenalectomized rats) at 32° C; Intact-Adrenalectomized at 37° C. In certain instances the design was modified as the experiment progressed and additional groups were introduced into the sequence. The values for LVWI in Table 1 were calculated by pooling all data. The conclusions reached are not different from those based

[4] If the resistance clamp was adjusted for a lower mean arterial blood pressure, the preparation expelled blood for an additional period.

[5] Under all experimental conditions of this study, right atrial pressure invariably increased as cardiac output decreased (See Fig. 2 for two typical experiments). For this reason no further reference to right atrial pressure will be made.

TABLE 1. AVERAGE LVWI OF ISOLATED HEART-LUNG

Group	Steroid added	Conc. μg./100 ml.	Average LVWI gram-Meters per 100 mg. heart	No. preparations	Standard Deviation	Coef. variation in %
Experiment A.[1] *Mean Arterial Blood Pressure, 80 mm. Hg; Temperature, 37° C*						
1-Intact-Intact[2]	—	—	444	12	196	44
2-Intact-Adrenx[3]	—	—	136	6	108	79
Experiment B. *Mean Arterial Blood Pressure, 110 mm. Hg; Temperature, 32° C.*						
3-Intact-Intact	—	—	951	12	343	36
4-Intact-Adrenx	—	—	491	12	152	31
5-Intact-Adrenx	B[4]	2	803	7	213	27
6-Intact-Adrenx	B	10	674	9	153	23
7-Intact-Adrenx	B	50	303	7	85	28
8-Intact-Adrenx	B	500	116	7	40	34
9-Intact-Adrenx	THF[5]	2	461	7	104	23
10-Intact-Adrenx	THF	10	488	5	96	20
11-Intact-Adrenx	THF	50	263	5	49	19
12-Intact-Adrenx	THF	500	282	5	56	25
13-Intact-Adrenx	S[6]	10	510	5	112	22
14-Intact-Adrenx	F[7]	10	867	3	168	19
Experiment C. *Mean Arterial Blood Pressure, 110 mm. Hg; Temperature, 37° C.*						
15-Intact-Intact	—	—	268	12	100	37
16-Intact-Adrenx	—	—	92	12	42	45
17-Intact-Adrenx	B[4]	2	102	13	54	53
18-Intact-Adrenx	B	10	232	12	93	40
19-Intact-Adrenx	B	50	88	13	36	41
20-Intact-Adrenx	B	500	38	12	17	46
21-Intact-Adrenx	THF[5]	2	96	7	29	31
22-Intact-Adrenx	THF	10	103	7	32	31
23-Intact-Adrenx	THF	50	90	7	27	30
24-Intact-Adrenx	THF	500	70	10	38	54
25-Intact-Adrenx	S[6]	10	101	7	27	26
26-Intact-Adrenx	F[7]	10	244	3	57	23
Experiment D.[8] *Mean Arterial Blood Pressure, 130 mm. Hg; Temperature, 37° C.*						
27-Intact-Intact	—	—	169	12	63	37
28-Intact-Adrenx	—	—	42	10	33	79
29-Intact-Adrenx	Aldo.[9]	5×10^{-4}	74	4	34	46
30-Intact-Adrenx	Aldo.	5.2×10^{-2}	198	4	38	19

[1] Data obtained from previous publication (1).
[2] Intact-Intact = heart lung preparation from intact rat perfused with blood from intact rats.
[3] Intact-Adrenx = heart-lung preparation from intact rat perfused with blood from adrenalectomized rats.
[4] B = Kendall's Compound B, Corticosterone, (Δ^4-pregnene-11β, 21-diol-3, 20-dione).
[5] THF = Tetrahydrocortisol, (pregnane-3α, 11β, 17α, 21-tetrol-20-one).
[6] S = Reichstein's Substance S, (Δ^4-pregnene-17α, 21-diol-3,20-dione).
[7] F = Cortisol (Δ^4-pregnene-11β,17α,21-triol-3,20-dione).
[8] Results obtained by Miss Kathyrn Ballard.
[9] Aldo. = Aldosterone, (Δ^4-pregnene-11β,21-diol-18-al-3,20-dione).

on individual studies conducted at different times within a period of three months.

In a series of eleven heart-lung preparations from rats housed at a relatively high and fluctuating environmental temperature (26.5 to 35° C) for a two-week period

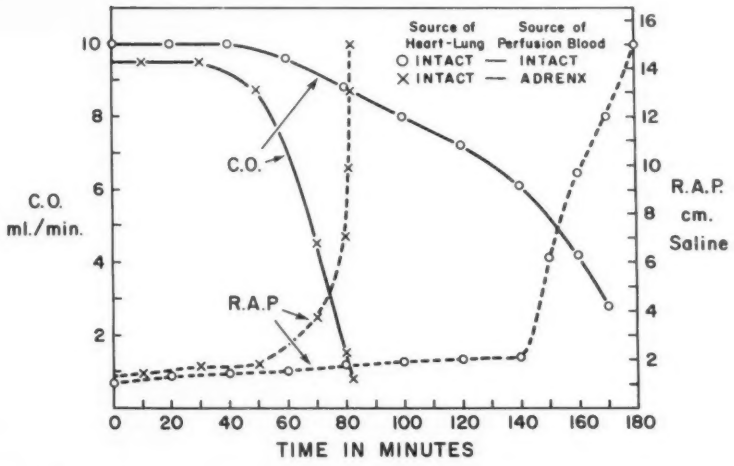

FIG. 2. Cardiac output (left ordinate) and right atrial pressure (right ordinate) plotted against time (abscissa) for two typical experiments. Mean arterial blood pressure, 110 mm. Hg.; perfusion temperature, 32° C. Area under each curve is related by a constant to LVWI. Data and calculation of LVWI for the Intact-Intact preparation follows:

Intact-Intact Preparation: MABP, 110 mm. Hg; perfusion temperature, 32° C

Time min.	CO ml./min.	Average CO[1] ml./min.	$C O_t$ ml.
0	10		
		10	200
20	10		
		10	200
40	10		
		9.9	198
60	9.8		
		9.7	194
80	9.6		
		9.0	180
100	8.4		
		8.1	162
120	7.8		
		7.3	146
140	6.8		
		5.7	114
160	4.6		
		3.8	38
170	3.0		
			$\Sigma(C O_t) = 1432$ ml.
171	0		
172	2.8[2]		

$$LVWI = \Sigma(CO_t)\ (MABP)\ (K)$$
$$= (1432)(110)(0.0136)$$
$$= 2142 \text{ gram-Meters}^3$$

[1] Average cardiac output is obtained by averaging two successive cardiac output readings.

[2] Despite maximum tension on the resistance clamp, the mean arterial blood pressure could not be maintained at the preselected level of 110 mm. Hg. Reduction in resistance for a pressure of 80 mm. Hg resulted in continued performance.

[3] Dry heart weighed 230 mg. LVWI may be expressed as gram-Meters per 100 mg. dry heart. In this case LVWI equals 2141 gram-Meters $\times$ 100/230 = 931 gram-Meters per 100 mg. dry heart.

LVWI was significantly less than that of heart-lung preparations from a series of rats housed at 25.5 ± 0.6° C. Respiratory infection as judged by sniffles, by rales, and, more definitively, by hemorrhagic consolidation of the exposed lungs, was associated with a markedly reduced performance of the heart-lung preparation.

The coefficient of variation (*standard deviation divided by the mean times 100*) of LVWI for the heart-lung preparations described in this study has been relatively uniform for a variety of experimental conditions (Table 1, Experiments B and C). The average coefficient of variation for Experiments B and C is 32% and the individual coefficients fall within a relatively narrow range (19 to 53%). On the other hand, the magnitude of the standard deviations of these same experiments parallels the absolute value of the LVWI (Figure 3). For these reasons the coefficient of variation has been accepted as the means by which to evaluate the degree of standardization attained. Another objective approach to the evaluation of the homogeneity of a group of rats is the range of the initial cardiac output. At a mean arterial blood pressure of 110 mm. Hg. and a perfusion blood temperature of 37° C, initial cardiac output ranged from 8.0 to 12.5 ml. per minute in a group of acclimatized, healthy rats. At the same temperature and pressure, initial cardiac output is usually 8.0 ml. or less per minute for heart-lung preparations from rats with a respiratory infection. Preparations exhibiting an initial cardiac output of 8.0 or less ml. per minute were not included in the tabulated averages (Table 1). If a relatively large number of preparations exhibit a low initial cardiac output, experiments are suspended until a new colony of healthy rats has been established.

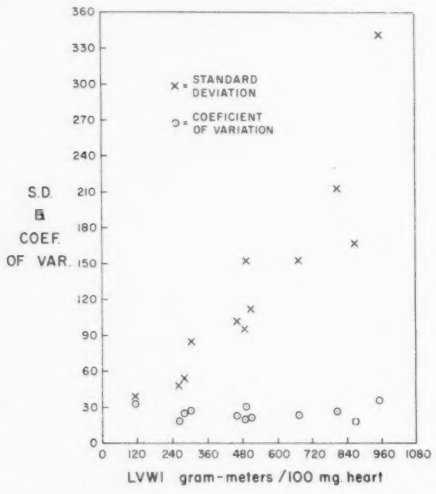

FIG. 3. LVWI plotted against standard deviation (×) and against coefficient of variation (⊙). Experiments B and C (Table 1) are represented.

RESULTS AND DISCUSSION

The early studies of Evans (2), of Starling and Visscher (3) and the more recent observations of Reissmann and Van Citters (4) have clearly demonstrated that the temperature of the perfusion blood and the resistance to flow exert profound influences upon the usual parameters employed to assess the functional capacity of the dog heart-lung preparation. As expected, and as will be illustrated by the data presented in the first two parts of this section, temperature and MABP significantly influence the work performance of the rat heart-lung. Maintenance of temperature and of MABP constant throughout the performance of the heart-lung established favorable conditions for evaluating the influence of the corticosteroids on this isolated preparation, which subject forms the body of the third part of this section of the paper.

1. Temperature of perfusion blood and performance of the heart-lung. In the case of Intact-Intact preparations, operating at 110 mm. Hg and a perfusion blood temperature of 32° C, LVWI equalled 951 ±343 gram-Meters (Table 1, Group 3). When the temperature of the perfusion blood was elevated to 37° C, other conditions remaining the same, LVWI was markedly reduced (268 ±100, Group 15). In the case of Intact-Adrenalectomized preparations, operating at 110 mm. Hg the same dramatic effect of temperature was observed (491 ±152, Group 4 at 32° C and 92 ±42, Group 16 at 37° C). Average heart rate was 322 ±6.6 (64 of the preparations in Experiment C, Table 1) when the perfusion blood was maintained at 37° C, and 225 ±8.9 (40 preparations of Experiment B) when the perfusion blood was

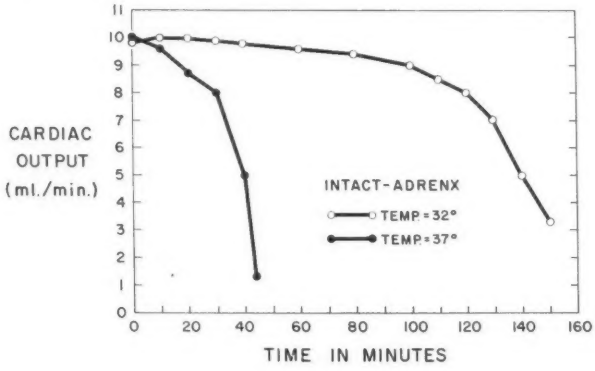

FIG. 4. Cardiac output plotted against time for two typical Intact-Adrenalectomized preparations, one perfused with blood at 37° C, the other perfused with blood at 32° C. MABP, equalled 110 mm. Hg.

maintained at 32° C. Since initial cardiac output was the same at the two temperatures, initial stroke volume was greater at the lower temperature. Figure 4 illustrates the subsequent course of events during two typical experiments, one at 32° C, the other at 37° C. It is obvious that cardiac output decreases more rapidly at the higher temperature. From the standpoint of standardization, it is obvious from the data that the temperature of the perfusion blood must be maintained within a relatively narrow range. In all experiments included in Table 1, temperature was maintained within ±1° C.

2. Peripheral Resistance (the Determinant of MABP) and Performance of the Heart-Lung. As stated in the section on methods, peripheral resistance was periodically adjusted to maintain MABP at a preselected level. LVWI was determined for three levels of pressure, 80, 110, and 130 mm. Hg. In both Intact-Intact preparations and Intact-Adrenalectomized preparations, LVWI decreased as pressure increased (Table 1, *cf.* Groups 1, 15, and 27; 2, 16, and 28). In Figure 5, the sequence of changes in cardiac output

with time for the three pressures studied is graphically presented. Obviously the MABP at which the rat heart-lung preparation operates has a pronounced influence on LVWI. In studies designed to test the influence of the corticosteroids, peripheral resistance was adjusted to maintain the pressure fixed at a preselected level for any given experiment.

3. *Corticosteroids and Performance of the Heart-Lung.*

a. Perfusion blood from intact and from adrenalectomized rats. In a previous study from this laboratory (1), it was demonstrated that the LVWI of heart-lungs from intact rats was influenced by the source of perfusion blood. At a temperature of 37° C and at a mean arterial blood pressure

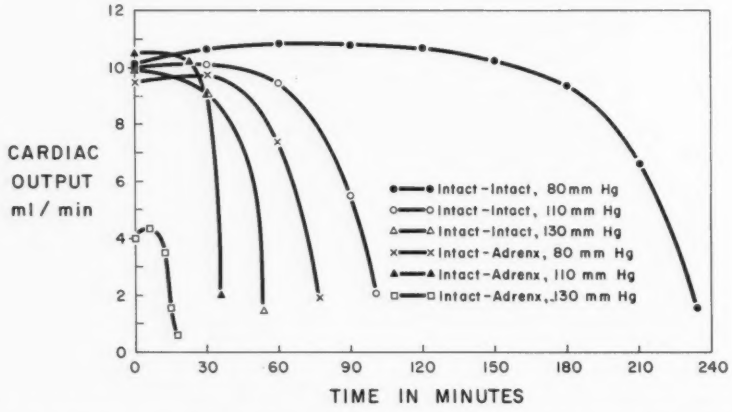

FIG. 5. Cardiac output plotted against time for a mean arterial blood pressure of 80, of 110 and of 130 mm. Hg (a typical experiment from each of the following Groups: 1, 2, 15, 16, 27 and 28, Table 1). Perfusion blood tempearture was 37° C for all preparations. Heart rate was not influenced by the level of the MABP.

of 80 or 110 mm. Hg, LVWI was less in preparations perfused with blood from adrenalectomized rats than in preparations perfused with blood from intact animals. The results of a new series at 37° C and a pressure of 110 mm. Hg are presented in Table 1 (*cf.* Groups 15 and 16) and confirm the results of the earlier study. The comparison has been extended to another temperature and to another pressure. At a perfusion temperature of 32° C and a pressure of 110 mm. Hg, blood from adrenalectomized rats was associated with lower LVWI than blood from intact rats (*cf.* Groups 3 and 4). At a temperature of 37° C and a pressure of 130 mm. Hg, blood from adrenalectomized rats was again observed to be associated with low LVWI (*cf.* Groups 27 and 28). In all instances cited above, initial cardiac output has been remarkable uniform and differences in LVWI, be they associated with changes in temperature

of perfusion blood, in mean arterial blood pressure, or in source of perfusion blood, represent differences in duration of function of the preparation. However, at 130 mm. Hg, the Intact-Adrenalectomized preparation in many instances exhibited a distinguishing feature, namely, relatively low initial cardiac output (Figure 5) and the low LVWI of this preparation may be ascribed to low initial cardiac output as well as to short duration of function.

These observations, which cover a fairly wide range of experimental conditions, clearly demonstrate that blood from adrenalectomized rats is deficient in a cardiotonic substance or contains a cardiac depressant. The results on addition of crystalline steroids (*vide infra*) indicate that at least one major factor responsible for the relative ineffectiveness of blood from adrenalectomized rats is a deficiency of corticosteroids.

b. Corticosteroids: structure activity relationship. The results of the present study confirm those of an earlier report from this laboratory (1) that corticosterone has a stimulatory action on the heart-lung preparation. Corticosterone, added to perfusion blood from adrenalectomized rats in a concentration of 10 μg. per 100 ml., increased LVWI of the Intact-Adrenalectomized preparation to a level not significantly different from that of the Intact-Intact preparation (*cf.* Groups 15, 16, and 18). It is of interest in this connection that chemical analyses of peripheral blood of the rat (5) indicate that corticosterone is present in a concentration of about 5 μg. per 100 ml. of blood.[6] Furthermore, cortisol (*cf.* Groups 15, 16, and 26) and aldosterone (*cf.* Groups 27, 28, and 30) were effective in elevating the LVWI of the Intact-Adrenalectomized preparation to that of the Intact-Intact preparation. Tetrahydrocortisol (Groups21, 22, 23, and 24) and Substance S (Group 25) showed no stimulatory action at the concentrations tested.Of the five steroids tested, those which exhibit biological activity in the heart-lung, exhibit biological activity in the whole animal. The data suggest that the α,β unsaturated group in ring A and the oxygen at C-11 are important in the exhibition of activity in the heart-lung. Obviously, the study must be extended to include a larger number of corticosteroids, and as will become clearer from the next section, must also be extended to include a relatively wide range of concentrations for any given steroid tested before any additional statements on structure activity relationship may be made.

c. Stimulatory and inhibitory concentrations of corticosterone: temperature dependence. Corticosterone was added to the perfusion blood of Intact-Adrenalectomized preparations which operated at a temperature of 37° C and a mean arterial pressure of 110 mm. Hg, (Groups 17, 18, 19, and 20). A concentration of corticosterone of 2μg. per 100 ml. of perfusion blood had

[6] After correction of the data of Guillemin *et al.* (5) for non-steroidal fluorescent substances and assuming that rat erythrocytes are free from corticosterone.

no significant effect on LVWI. At 10 μg. per 100 ml., the work performed by the heart was not significantly different from that of an Intact-Intact preparation. A concentration of 50 μg. per 100 ml. resulted in an LVWI not significantly different from that of the Intact-Adrenalectomized preparation to which no steroid was added (*cf.* Groups 16 and 19). Therefore, corticosterone in a concentration of 50 μg. per 100 ml. is inhibitory when compared to a concentration of 10 μg. per 100 ml. When the concentration of corticosterone was increased to 500 μg. per 100 ml., LVWI was actually less than that of the Intact-Adrenalectomized preparation to which no steroid was added (*cf.* Groups 16 and 20). These observations emphasize the importance of surveying concentration before making a final statement on the influence of a substance on a biological system, an approach that has been effectively championed by Ingle (6). These observations may explain, in part at least, the confusion in the literature as to the action of corticosteroids on the isolated heart. Emele and Bonnycastle (7) recognized the importance of concentration and have described stimulatory and inhibitory actions for the same steroid on the cat papillary muscle depending upon the concentration employed.

Tetrahydrocortisol was tested at concentrations of 2, 10, 50, and 500 μg. per 100 ml. perfusion blood in the Intact-Arenalectomized preparation at 37° C and a mean arterial pressure of 110 mm. Hg (Groups 21, 22, 23, and 24). No stimulatory action was exhibited at any of these concentrations; 500 μg. per 100 ml. exerted a slight, and probably insignificant, inhibitory effect.

Corticosterone was tested for its effect on the Intact-Adrenalectomized preparation operating at 32° C and a mean arterial pressure of 110 mm. Hg. Whereas, at 37° C a concentration of 2 μg. per 100 ml. was ineffective, at 32° C this concentration of steroid increased the LVWI of the Intact-Adrenalectomized preparation to a level not significantly different from that of the Intact-Intact preparation (*cf.* Groups 3, 4, and 5). Furthermore, at the low temperature, 2 μg. per 100 ml. of corticosterone was at least as effective as 10 μg. per 100 ml., and 50μg. as well as 500 μg. per 100 ml. was definitely inhibitory (Groups 6, 7, and 8). These observations indicate that both the stimulatory and inhibitory concentrations of corticosterone in the heart-lung preparation are shifted in the same direction, *i.e.*, to lower values, as the temperature is reduced.

Tetrahydrocortisol was inactive at 2 and at 10 μg. per 100 ml. when the temperature of the perfusion blood was 32° C (Groups 9 and 10), whereas a definite inhibitory effect was exhibited at 50 as well as at 500 μg. per 100 ml. It is to be noted that at 37° C, 50 μg. had no effect and 500 μg. exerted at most a slight inhibitory action. The reason for the increased "toxicity" with reduction in temperature exhibited by tetrahydrocortisol, a steroid which is metabolically inactive in the whole animal, is obscure.

The temperature dependence of the stimulatory concentration of cortico-

sterone in the heart-lung is of considerable interest. Whether factors other than temperature influence the concentration-response relationship must await further study. For example, the stimulatory and inhibitory concentrations of corticosterone should be determined in two preparations operating at the same temperature but at different mean arterial pressures. It appears that in the whole animal the concentration of corticosteroids required to maintain well-being is not fixed but varies according to the state of the internal and/or the external environment (8). The need for corticosteroids increases in hyperthyroidism, in infection, during exercise and during exposure to abnormal environmental temperatures. Obviously, it would be of some importance to examine the possibility that the need for corticosteroids by an isolated system such as the heart-lung preparation varies according to the metabolic state or the environment of the cells.

CONCLUDING REMARKS

The standardization of the rat heart-lung preparation has been developed to a point where application to bioassay of cardiotonic or cardiac inhibitory substances appears feasible. A concentration-response relation of the type reported here for corticosterone is being extended to include aldosterone. Cardiotonic agents, in particular the cardiac glycosides, may be evaluated in the rat heart-lung.

Inadequacy of cardiovascular function, which is a dominant feature of adrenocortical insufficiency, has been ascribed to failure of the myocardium (9), to poor vasomotion of the arterioles (10), and to increased permeability of the capillaries (11). The results of the present study offer strong support to the thesis that hypofunction of the myocardium is at least one of the factors resulting in cardiovascular collapse in the crisis of adrenocortical insufficiency.

The results of this study clearly demonstrate a stimulatory action of corticosterone, cortisol, and aldosterone on the rat heart-lung preparation. Other investigators have reported stimulatory or inhibitory effects of steroids in simpler systems, namely, heart strips, heart papillary muscles and perfused Langendorff preparations (7, 12, 13). In certain instances, the concentration of steroid employed, the establishment of specificity of steroid action, or the use of appropriate controls have raised doubts as to the physiological significance of data obtained from these simpler systems.

The mode of action of the corticosteroids in increasing the LVWI of the heart-lung preparation remains unknown. Actually, the site of action cannot be definitely localized to the myocardium on the basis of the present study. It is possible that the corticosteroids exert their effect in whole or in part by way of the lung. Preliminary studies with an isolated heart preparation (perfusion of the left atrium; pulmonary vessels out of the circuit) suggest that the corticosteroids have a direct action on the myocardium. The fact that aldosterone is much more potent than corticosterone suggests

that electrolyte balance may be a profitable area to study in elucidating mode of action. Experiments are planned to examine the possibility that alterations in water and electrolyte balance are associated with the stimulatory or inhibitory actions of the corticosteroids on the myocardium. However, there is no compelling reason to assume that the mode of action of the corticosteroids on the heart is related to the metabolic actions of these substances derived from whole animal studies.

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HYPERGLYCEMIA CAUSED BY THE ORAL ADMINISTRATION OF GLUCOSE IN TURTLES

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ABSTRACT

1. Glucose was given orally, at a dose of 1–2 gm./kg. per day for 30 days, to two groups of turtles (*Chrysemys d'orbignyi*); one group was treated during the summer, the other during the winter. Hyperglycemia was produced in the animals of both groups; the mean blood sugar values attained during the winter were higher (1:140) than during the summer (193).

Histological examination of the pancreas of these animals showed an increase in the number and size of the islets of Langerhans, with degranulation and hydropic degeneration (glycogenic infiltration) of the beta cells.

The hyperglycemia and the histological lesions disappeared when treatment was discontinued.

THE present experiments were carried out in the course of a study of the pancreatic lesions caused by elevation of the blood sugar in various animals.

Woerner (1), giving glucose intravenously to guinea pigs, did not obtain a noticeable rise in blood sugar but did observe a diminution of the granulation of the beta cells and an increase of the islet tissue followed eventually by degenerative changes. Also in guinea pigs, Gomori, Friedman and Caldwell (2) observed hyperglycemia followed by degranulation of the beta cells after repeated intraperitoneal administration of glucose. Because the beta cell granules reappeared after the administration of glucose was stopped and the blood sugar returned to normal, they concluded that there had been an increase of insulin secretion in response to the hyperglycemia.

More recently, Ingle (3) force-fed rats with a high carbohydrate diet, obtaining elevated blood sugars and glycosuria; no histological observations were made on the pancreatic islets of these rats.

Dohan and Lukens (4) obtained permanent diabetes in one previously normal and two partially depancreatized cats after intraperitoneal injection of glucose for 38, 52 and 55 days, respectively.

At the suggestion of Professor Bernardo Houssay, we repeated these experiments in turtles, to see if we could obtain similar results, such as hyperglycemia, damage of the beta cells and, finally, permanent diabetes.

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MATERIAL AND METHODS

Adult *Chrysemys d'orbignyi* turtles of both sexes were used. They were maintained on a fish diet, which was available *ad libitum* except during the fasting period prior to each blood sugar determination. The body weights ranged from 1,200–3,000 gm. including the shell, which comprises about 30% of the total.

The glucose, dissolved in distilled water (1 gm./ml.), was introduced into the stomach by means of a urethral catheter, being careful to keep the maxillars separated to protect the catheter. This was done once a day and at the same hour. For each group there was a corresponding group of controls which received only distilled water (1 ml./kg.).

Part of the experiment was done in the summer, another part in the middle of the winter. The animals were kept outside in tanks with running water at temperatures of approximately 20.8°–30.5° C in summer and 9.8°–18.1° C in winter.

Thus, we had two groups, one in the summer and one in the winter, each being given glucose at the rate of 1 to 2 gm./kg. per day for 30 days. Furthermore, we had a third group in which we tried to produce permanent diabetes in this fashion; this was done during the winter because hyperglycemia was more easily obtained then. In this group the glucose dose varied between 0.5 and 5 gm./kg.

The blood samples were taken after a fasting period of approximately 15 hours, and the blood sugar determined according to the Somogyi-Nelson method (5).

RESULTS

During the summer (Table 1), the daily administration of 1 gm. of glucose per kg. body weight for 23 days, followed by 2 gm./kg./day for 7 days more, raised the animal's blood sugar moderately, from about 93 mg.% in the controls to 193 mg.% in the treated series; it was noticed that there was a tendency toward higher blood sugars in the females.

TABLE 1

Blood sugar values for the turtle *Chrysemys d'orbignyi*. Two or three sugar analyses were obtained for each turtle in the summer series; (1) between the 10th and 23rd days of force-feeding glucose, (2) between the 24th and 38th days, (3) 10 days after discontinuing glucose feeding; for the winter series two analyses were obtained: (1) between the 15th and 23rd days of treatment, and (2) between the 24th and 30th days. The turtles were kept at environmental temperatures of approximately 21–31° C (mean values of the minimum and maximum) in the summer and 10–18° C in the winter.

Season	Treatment	Blood sugar	
		Analysis period	Value, mg.%
Summer Exp.	Control, no glucose given	(1)	91 ± 11 (6)*
		(2)	95 ± 18 (5)
	Glucose fed, 1 gm./kg./day from 1st to 23rd day, 2 gm./kg./day from 24th to 30th day	(1)	193 ± 25 (12)
		(2)	193 ± 32 (12)
		(3)	63 ± 11 (6)
Winter Exp.	Control, no glucose given	(1)	80 ± 7 (6)
		(2)	76 ± 3 (6)
	Glucose fed, 2 gm./kg./day from 1st to 23rd day, 1 gm./kg./day from 24th to 30th day	(1)	443 ± 60 (10)
		(2)	1140 ± 306 (7)

* Mean ± standard error; the number of animals is given in parentheses. Owing to death of some animals during treatment, the number during the second or third period may be less than during the first.

It is clear that administration of similar doses of glucose during the winter (Table 1) produced much higher blood sugars, up to a mean of 1,140 mg.% (470–2,380) in the treated series as compared to 76 mg.% (65–80) in the corresponding controls. The winter group received 2 gm./kg./day of glucose during the first 23 days and 1 gm./kg./day during the following 7 days. Glucose was found in the urine of these animals. Towards the end of the experiment the animals showed muscular hypotonia, and all died, even when treatment had been stopped.

Mortality varied according to the dosage and the season of the year. With a dose of 1 gm./kg. during the summer, the mortality was 50% after 20 to 30 days of treatment. In winter, with a dose of 2 gm./kg./day, the mortality was 60% after 15–20 days of treatment, and 100% after 30 days.

The histological examination of the pancreas was done by Dr. Cardeza (6), who found an increase in number and size of the islets of Langerhans and degranulation and hydropic degeneration (glycogenic infiltration) of the beta cells. He found these lesions to be related to the severity of the hyperglycemia and reversible; in most animals sacrificed 30 days after discontinuing the treatment, the lesions had disappeared; two turtles still showed residual lesions.

Two series of experiments were carried out with the intent of producing

TABLE 2

Dosage schedule and blood sugars for turtles *Chrysemys d'orbignyi* force-fed with glucose solutions. The glucose was administered daily and one blood sugar was obtained for each turtle in each of the dosage periods. The dosage for each individual turtle was adjusted for each period in an effort to maintain an intermediate degree of hyperglycemia, the average daily dose per turtle for the whole experiment being about 2 g/kg in the first experiment and slightly less in the second. The experiments were performed during the winter at environmental temperatures of approximately 11–19° C (mean values of the minimum and maximum).

Series No.	Period of treatment, days	Average glucose dose and range for all turtles, gm./kg./day	Blood sugar, mg.%
1	1– 4	5.0	231 ± 33 (10)*
	5– 8	2.4 (0–5)	259 ± 81 (9)
	9–11	1.6 (0–3)	316 ± 92 (8)
	12–14	1.4 (0–4)	242 ± 82 (5)
	15–16**	1.5 (0–3)	586 (1)
2	1– 4	1.9	61 ± 5 (8)
	5–11	1.4	71 ± 10 (8)
	12–23	2.4	209 ± 51 (8)
	24–26	1.8 (1–3)	339 ± 41 (4)
	27–31***	1.2 (0–3)	559 ± 88 (7)

* Mean ± standard error; the number of animals for which blood sugars were determined is given in parenthesis.

** One turtle of this experiment died on the 46th day, after exhibiting blood sugars of 361 on the 25th day, 1160 on the 39th, and 756 on the 46th; the glucose dosage was 2 g./kg./day from 23–26th, 0.5 from 27–30th, 0 on 31 and 32nd, 1 from 33–39th days, zero thereafter. A second turtle of this series died on the 43rd day after blood sugars of 42 on the 25th, 443 on the 39th, and 278 on the 41st day; the glucose dosage was 3 g./kg./day from 23–38th, 2 on 39th, 0.5 g./kg./day thereafter. The remaining 3 animals died before the 24th day.

*** Seven turtles of this experiment died between the 32 and 37th days of treatment without a record of the terminal blood sugars.

permanent diabetes (Table 2). In the first series the initial dose of glucose was large and progressively decreased; in the second series the initial dose was made smaller with the hope of getting maximal survival. In both series it was necessary to adjust the dosage of glucose in accordance with the blood sugar levels, in order to obtain a persistent but moderate hyperglycemia. No permanent diabetes resulted in any case, possibly because the period of treatment was too short. All animals died within 42 days.

DISCUSSION

As in other animal species, the administration of a certain amount of glucose raises the fasting blood sugar in turtles and causes, secondarily, the appearance of lesions in the beta cells. These lesions are reversible, retrogressing after the interruption of treatment (see (1)).

Under these conditions of experiment, it was never possible to obtain permanent diabetes, possibly because the animals could not withstand the handling to which they were submitted daily, i.e. immobilization and introduction of the catheter; the mortality in the controls was also high, even though they received only distilled water instead of glucose.

The highest blood sugar values were obtained during the winter time, possibly because, due to the low temperature and the subsequent lowering of the metabolism in these poikilothermic animals, the consumption of glucose was reduced.

2. An attempt to obtain permanent diabetes failed in a third groups of turtles, possibly because of the high mortality arising from the requisite daily immobilization and catheterization of the animals.

Acknowledgment

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DECREASED GONADOTROPHIC HORMONE CONTENT OF THE ANTERIOR PITUITARY GLAND IN DOGS WITH HYPOTHALAMIC LESIONS[1,2]

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ABSTRACT

Assays for interstitial cell stimulating hormone and follicle stimulating hormone activity have been performed on the pituitaries of five male dogs with lesions in the posterior median eminence and the pituitaries of ten castrate male dogs with lesions in various parts of the hypothalamus. All dogs with destruction of the posterior median eminence showed depressed ICSH and FSH values. A lesion in the anterior median eminence in one castrated dog caused a lesser depression of pituitary content of these hormones; other lesions studied had no effect. The presence of a lowered FSH and ICSH content in the pituitary was not correlated with depressed thyroid or adrenal function or with the presence of diabetes insipidus. It is suggested that lesions in the posterior median eminence in the dog selectively inhibit the production and secretion of both ICSH and FSH.

IN THE dog, lesions of the posterior median eminence of the hypothalamus are associated with atrophy of the testes and prostate without effect on thyroid or adrenocortical function (1, 2, 3). This atrophy resembles that seen after hypophysectomy and is probably due to a diminished secretion by the pituitary of both follicle stimulating hormone (FSH) and interstitial cell stimulating hormone (ICSH) (3). The testicular atrophy seen in these animals with hypothalamic lesions could conceivably be due to inhibition of ICSH secretion alone, since the administration of ICSH to hypophysectomized male rats leads to maintenance of seminiferous tubules (4) and there is doubt about the physiological role of FSH in the male (5). Data on changes in the pituitary content of these gonadotrophins would therefore be of considerable interest. Accordingly, we have investigated the gonadotrophin content of the anterior pituitary of intact and castrate dogs with hypothalamic lesions.

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[2] These data were submitted by the senior author in partial fulfillment of the requirements for the Ph.D. degree in Animal Physiology at the University of California.

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MATERIALS AND METHODS

Male mongrel dogs with semen containing a high concentration of motile sperm were used in this study. The methods of semen collection and evaluation have been described elsewhere (3). In 15 dogs, electrolytic lesions were placed in various parts of the hypothalamus by the technique of Hume and Ganong (6). The testes were removed at the same time in 10 of these animals.

Twenty-one days postoperatively, thyroidal uptake of radioactive iodine (7) and urine volume were measured in order to evaluate the effect of the operations on thyroid function and development of diabetes insipidus. Diabetes insipidus was said to be present when urine volume exceeded 700 ml. per day for two consecutive days (2). Forty-two days postoperatively, 5 of the castrate dogs with lesions were anesthetized with pentobarbital and their adrenal venous output of hydroxycorticoids in response to surgical trauma was measured by methods which have been described elsewhere (3). Seven castrated dogs without hypothalamic lesions served as controls for the studies of thyroid and adrenal function and urine output.

All fifteen animals with lesions were sacrificed 37 to 47 days postoperatively by the intravenous injection of a lethal dose of pentobarbital, and the pituitaries were removed within 4 minutes after the injection. The anterior lobe of the pituitary was dissected from the posterior lobe, weighed (in 8 animals with lesions), and immediately frozen for subsequent bioassay. The adrenal and thyroid glands were weighed, fixed in Bouin's solution, sectioned, and stained with hematoxylin and eosin. The hypothalamus was fixed in formalin and subsequently serially sectioned at 16 micra. Every tenth section was stained with hematoxylin and eosin. The location of the lesions was determined, and in each case, the lesions were reconstructed on a diagram of a sagittal section of the dog hypothalamus.

The pituitaries from 10 of the castrated dogs with lesions in various parts of the hypothalamus were individually bioassayed in hypophysectomized rats for their content of FSH and ICSH. The development of the follicles (8) and the appearance of the interstitial cells (9) of the ovary of the assay rats were used as the criteria for evaluating the gonadotrophic hormone content. Uterine and ovarian weights were also recorded. Details of the assay and the values in normal and castrate male dogs have been reported elsewhere (10). In this study, the individual anterior lobes were homogenized in saline and injected subcutaneously over 4 days in total doses of 0.8 and 0.2 of the anterior lobe, each dose into one assay rat. These doses were selected because they were well above the minimum effective doses (MED) for FSH (0.06 pituitary) and ICSH (0.125 pituitary) in the pituitaries of castrated dogs (10). The pituitary from one of the castrated dogs with a lesion was assayed at a total dose of 0.35 anterior pituitary gland. The anterior pituitaries of non-castrated animals with lesions were pooled and tested at total doses of up to three glands, a figure which is twice the MED for FSH and 12 times the MED for ICSH present in the pituitaries of normal male dogs.

RESULTS

The pituitaries from the 5 non-castrate dogs with posterior median eminence lesions and gonadal atrophy were negative for FSH and ICSH at a total dose of 3.0 anterior lobes. The average weight of the pituitaries from these dogs with lesions was 4.5 ± 0.6 mg./kg. body weight (mean $\pm$ standard error of the mean).

The data for the individually assayed pitulitaries of the castrate dogs with lesions are shown in Table 1. The ovarian and uterine weight of the recipient rats has been included to give additional information on the pres-

TABLE 1. EFFECT OF HYPOTHALAMIC LESIONS ON PITUITARY GONADOTROPHIN CONTENT OF CASTRATE DOGS*

Donor dog				Recipient rat				
	Pituitary weight			Total dose (fraction of pituitary injected)	Ovarian weight (mg.)	Uterus weight (mg.)	Ovarian follicle stimulation	Ovarian interstitial tissue
Dog No.	(mg.)	(mg./kg. body weight)	Lesion Location					
21	32.9	2.8	Post. ME**	0.2	8.7	33.2	Absent	Deficient
				0.8	6.8	28.0	Absent	Deficient
22	25.6	2.6	Post. and mid ME	0.2	8.2	35.5	Absent	Deficient
				0.8	9.0	23.8	Absent	Deficient
18	26.5	1.8	Post. ME, Post. tuber.	0.2	8.2	36.4	Absent	Deficient
				0.8	6.0	26.2	Absent	Deficient
32	33.8	2.9	Post. tuber, Post. ME	0.2	6.4	36.6	Absent	Deficient
				0.8	7.8	29.9	Absent	Deficient
8	21.0	1.9	Post. and mid ME	0.2	6.6	32.2	Absent	Deficient
				0.8	8.7	31.2	Absent	Deficient
2	37.6	2.6	Post. ME	0.2	5.0	21.6	Absent	Deficient
				0.8	4.0	17.6	Absent	Deficient
12	—	—	Post. ME	0.2	8.0	35.0	Absent	Deficient
				0.8	9.4	35.8	Absent	Deficient
19	49.2	3.0	Ant. ME—post. Optic area.	0.2	7.2	35.6	Absent	Deficient
				0.8	20.2	77.4	Present	Repair
11	48.8	4.0	Post. tuber Part of Pre-Mammillary n.	0.35	23.7	85.6	Present	Repair
7	—	—	Small unilat. Lesion in dorsal Hypothalamus.	0.2	12.0	47.0	Present	Repair
				0.8	38.5	137.4	Present	Repair
				0	7.2±0.3***	22.3±1.2	Absent	Deficient

* Each dose level was tested on one hypophysectomized assay rat.
** Median Eminence.
*** Mean ± Standard error of the mean in 33 uninjected control hypophysectomized rats.

ence or absence of ovarian stimulation. On histological examination of the hypothalamus, bilateral destruction of the posterior median eminence was found to be present in 7 dogs. The occurrence of a lower uterine weight in the recipient rats receiving 0.8 pituitary from 6 of these 7 dogs with posterior median eminence lesions is apparently fortuitous; the mean uterine weight in these rats is not significantly different from the value in those receiving 0.2 pituitary ($P > 0.1$), and ovarian weights did not differ in this fashion. The average anterior pituitary weight in these dogs was 2.4 ± 0.2 mg./kg. At both levels tested the pituitaries of these dogs showed no evidence of FSH or ICSH activity.

In two cases (Dogs no. 7, no. 11), the median eminence was intact; in both instances, positive responses for both FSH and ICSH were present at the lower dose tested, 0.2 of the gland. One of these dogs (no. 7) had a small unilateral lesion above the median eminence; the other (no. 11) showed discrete destruction of the posterior tuberal region just behind the infundibular stem. In another dog (no. 19), the lesion involved the anterior portion of the median eminence. The pituitary of this animal was negative for FSH and ICSH at the 0.2 pituitary level, and positive for both at the 0.8 level.

The data on thyroid and adrenal function and urinary output in the castrate animals with lesions are presented in Table 2. In the animals with

TABLE 2. EFFECT OF HYPOTHALAMIC LESIONS ON OTHER ENDOCRINE GLANDS IN CASTRATE MALE DOGS

Dog No.	I^{131} uptake (Per cent of injected dose)	Left adrenal weight (mg./kg.)	17-OH corticoid output (μg./min.)	Diabetes insipidus (Urine volume 700 ml./day for two days)
1. Castrate dogs with hypothalamic lesions				
21	20.3	38.1	10.4	Yes
22	22.5	57.0	3.2	No
18	18.9	45.3	—	No
32	15.3	78.6	—	Yes
8	40.3	44.4	4.6	No
2	21.5	41.4	1.9	Yes
12	4.8	63.5	—	Yes
19	13.6	68.2	14.5	No
11	24.1	50.6	—	Yes
7	14.8	58.8	—	No
2. Castrate dogs without lesions				
56	17.2	63.4	—	No
63	17.5	58.6	—	No
69	19.9	53.7	—	No
1	41.3	59.8	7.6	No
3	33.8	95.6	16.9	Yes
25	18.3	51.1	10.1	No
35	37.4	64.7	13.0	No

depressed pituitary gonadotrophin content, thyroid I^{131} uptake was below the 95% confidence limits for castrate controls with no lesions in only one case, and the adrenocortical hydroxycorticoid output below these limits in two of 5 animals tested. Mean adrenal weight was not significantly different from castrate control animals, and adrenocortical histology was normal in all cases. The histological appearance of the thyroid glands was essentially normal. Diabetes insipidus was present in four of the animals with low pituitary gonadotrophins, but absent in three. There was, therefore, no correlation between any of these parameters and depressed pituitary gonadotrophin content. Hydroxycorticoid output in the castrate control animals without lesions was slightly higher and thyroidal radioactive iodine slightly lower than the values encountered in intact male dogs (3, 7), but the differences were not statistically significant. One of the castrate control animals (no. 3) had an unexplained polyuria, but study of serial sections of the brain failed to reveal any abnormality of the supraoptico-hypophyseal system.

DISCUSSION

The minimum effective dose (MED) of normal male dog anterior pituitary is 1.5 gland for FSH and 0.25 of a gland for ICSH (10). Since the pooled pituitaries of the 5 non-castrate dogs with lesions were negative for FSH and ICSH at a total dose of 3.0 pituitaries, the pituitary content of both these gonadotrophins was clearly depressed. The mean weight of the pituitary glands in these animals with lesions was slightly higher than the value (3.6 ± 0.4 mg./kg.) found in normal control dogs (10).

In otherwise normal dogs, 4 to 6 weeks after castration, the values for FSH and ICSH were 0.06 and 0.125 of a gland respectively; mean pituitary weight in such animals was 3.5 ± 0.6 mg./kg. (10). In the 7 castrate dogs with lesions of the posterior median eminence, the individually assayed pituitaries were all negative for both FSH and ICSH at 0.8 of a gland. The decrease in gonadotrophin content is therefore at least twelve-fold for FSH and at least 6-fold for ICSH, and is too great to be attributed to the slight decrease in the pituitary weight seen in some of these animals.

There are few data available on the effect of hypothalamic lesions on pituitary gonadotrophin content. Clegg and Ganong (12) have reported that following ventral hypothalamic lesions in female sheep, the pituitary FSH content is depressed without any change in ICSH content. Bogdanove *et al.* (13) reported a decreased total gonadotrophin content in the anterior pituitary of rats with testicular atrophy following hypothalamic lesions. The bioassay was based on the increase in uterine weight of recipient immature female rats injected with pituitary tissue. In some animals with lesions but without gross testicular atrophy, a moderate decrease in pituitary gonadotrophins was also present. Histologically normal gonadotrophs were present in the pituitaries of these animals. The authors suggested that the gonadotrophin content of the pituitary is a more sensitive index of gonadotrophin secretion than pituitary morphological changes. In the light of these observations, it is of interest that we found moderately decreased gonadotrophin values in one dog with a lesion in the anterior median eminence. Non-castrate male dogs with similar lesions show slight but significant decreased in semen count, sperm motility and ejaculation response (3).

The area of the hypothalamus destroyed in the dogs with an extremely low pituitary gonadotrophin content is the same as that reported to be concerned with gonadal and prostatic maintenance (3). Therefore, it is reasonable to conclude that destruction of the posterior median eminence in the male dog leads to a diminution in both the production and the secretion of pituitary ICSH and FSH.

Posterior tuberal lesions block copulation induced ovulation in cats and rabbits (14). Hume (15) has reported atrophy of the gonads in the male dog following lesions which are slightly more posterior than those reported here. However, it is interesting that in one animal in the present series with a lesion which completely destroyed the posterior tuberal region up to the pituitary stalk (no. 11), no depression of pituitary gonadotrophin content was seen.

It is possible that the posterior median eminence may represent a final common pathway to the anterior pituitary, and that other areas of the hypothalamus are concerned with the regulation of one or the other of these gonadotrophins. There is evidence (16, 17) that an area near the paraventricular nucleus has selective effects on ICSH secretion in female rats.

No evidence exists for a homologous area in the male, but none of the lesions in the present series destroyed this anterior region.

There is some evidence that depressed thyroid function inhibits gonadotrophic activity (18). However, the present results could not be secondary to depressed TSH secretion because in all but one dog (no. 12) radioactive iodine uptake and thyroid weight and histology were normal.

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ULTRASTRUCTURE AND FUNCTION IN NEUROHYPOPHYSIS OF THE TOAD[1]

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ABSTRACT

An electron microscope study was carried out on hypothalamic portions of the preoptic-neurohypophyseal tract and in the infundibular process of toads in normal water balance or treated experimentally to produce release of the posterior pituitary hormones.

Axons containing neurosecretory granules have been observed in the hypothalamus (region I) in the hilum of the infundibular process (region II), near the capillaries (region III) and at the ending proper (region IV).

The neurosecretory granules, that are formed within special dense vesicles, have a high electron density and are surrounded by an enveloping membrane. Their size increases from 620 Å in region I to 1350 Å–1500 Å in regions II and III.

At the ending (region IV) a second component appears which is similar to the synaptic vesicles described by De Robertis and Bennett (1954). The mean size of the vesicles is of 420 Å and the histograms show no overlapping between these components. Within the axons of region I to III neuroprotofibrils and finer neurofilaments are observed. In all parts of the axons there are bizarrely shaped mitochondria that bear no relationship to the neurosecretory material. In chronically dehydrated animals the neurosecretory granules disappear from the endings but persist in other regions of the axon. At the endings the synaptic vesicles remain and appear to increase in number after chronic dehydration.

In acutely dehydrated animals (injection of hypertonic NaCl) most neurosecretory granules disappear and only some empty membranes are observed at the endings. In this case also the synaptic vesicles seem to decrease in number. Ether anesthesia produces a massive release of neurosecretion and vacuolization of the nerve endings.

The concept of axoplasmic flow of neurosecretion is discussed on the basis of our findings. It is concluded that in addition to the streaming of secretion there should be a progressive synthesis of material along the axon.

The probable mechanisms of release are discussed and compared with those in other neurohumoral secretory processes. A hypothesis is advanced about the possible role played by the synaptic vesicles in the function of neurosecretory nerve fibers.

THE general structure of the neurohypophysis has been elucidated by use of histological techniques. It is now generally admitted that the main body of this gland consists of unmyelinated nerve fibers whose per-

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icarya are in several hypothalamic nuclei, particularly the supraoptic and paraventricular. These nerve fibers are intermingled with special glial cells called pituicytes and end on the blood capillaries by means of enlarged endings. In the *opossum* which is a very favorable material because of the anatomical organization of the gland, Bodian (1) distinguished a *hilar portion* containing the branches of the hypothalamic-hypophyseal tract and a *palisade region* in which the enlarged endings are perpendicularly oriented toward the connective and vascular septa that divide the gland into lobules. These hypothalamico-hypophyseal fibers contain the typical neurosecretory material that can be also found in the pericarya with selective staining techniques such as the Gomori chrome-alum-hematoxylin (see 2 and 3).

The electron microscope has so far been applied to the neurohypophysis of several mammals (4, 5, 6, 7, 8), to birds and reptiles (9 and 10).

These studies have revealed the presence of a neurosecretory material along the axons and at the terminals composed of dense granules of about 0.1 μ. In addition Palay (5) and Green and Maxwell (8) have described another component composed of pale granules or vesicles whose nature is discussed. The origin of this neurosecretory material has not been completely elucidated. For example Green and Maxwell (8) consider that the secretory granules are formed by the mitochondria found along the axons and Bargmann and Knoop (6) maintain that it is not possible to exclude the mitochondrial origin of the granules.

To our knowledge the neurohypophysis of amphibia so far has not been studied with the electron microscope. In this case there is only one neurosecretory nucleus in the hypothalamus—the preoptic nucleus—which is characterized by a rich vascular bed and the presence of neurosecretion. The axons can be followed histologically from the preoptic nucleus through the hypothalamico-neurohypophyseal tracts to the endings on the large capillaries of the infundibular process (Fig. 1). In the toad *Bufo arenarum* Hansel, the neurosecretory material is abundant and accumulates around the capillaries.

In the toad, as in other species, dehydration produces a diminution of the neurosecretory material and at the same time the antidiuretic activity of the tissue decreases (11).

In this work the fine structure of the neurohypophysis of the toad *Bufo arenarum* Hansel, has been studied with improved methods of fixation and high resolution microscopy. The neurosecretory axons have been followed from the hypothalamus and through the hilar regions toward the final ending on the capillaries. In addition to the neurosecretory material, a vesicular component similar to the synaptic vesicles has been found in the terminal region of the nerve endings. These two components have been studied in animals with the normal water balance and in experimental

conditions known to release, acutely or chronically, the hormones of the neurohypophysis.

TECHNIQUES

The material was from adult male toads (*Bufo arenarum* Hensel) maintained in captivity and sacrified in different seasons. Three main procedures were followed for the extraction of the tissue: a) Rapid decapitation without anesthesia and opening of the skull through the base, b) extraction after ether anesthesia which is known to produce a massive liberation of antidiuretic hormone (12), c) extraction after anesthesia with 30% alcohol injected intraperitoneally or by a gastric tube. This treatment does not produce hormone liberation (13).

After dissection of the hypothalamic and neurohypophyseal regions small pieces of tissues were taken from the preoptic-neurohypophyseal tract near the preoptic nucleus and from the infundibular process of the neurohypophysis. In Figure 1 is indicated a diagram of the main anatomical disposition of this region in the toad and approximately the zones which were then studied under the electron microscope.

Fixation was carried out in 1% osmium tetroxide dissolved in Periston (Bayer), a plasma substitute containing polyvinylpyrrolidone and ions. The fixation in Dalton's osmium tetroxide-potassium bichromate fluid did not give good results. After embedding in butyl and methylmetacrylate (9:1) the sections were made with a Porter-Blum

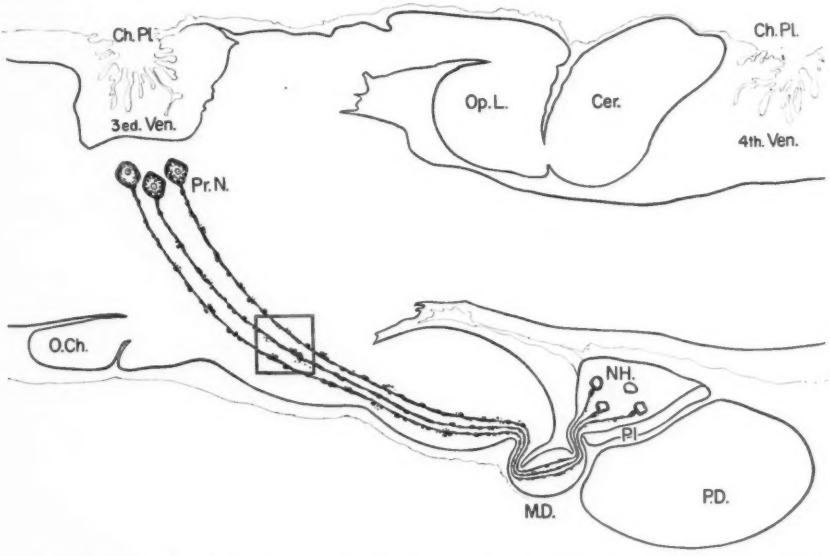

FIG. 1. Diagram of the hypothalamico-neurohypophysial system in the toad *Ch Pl* choroid plexus, *cer* cerebellum, *op. l.* optic lobe, *3rdven* third ventricle, 4thven fourth ventricle, *och* optic chiasma, *PrN* preoptic nucleus, *NH* infundibular process of the neurohypophysis, *pD* pars distalis, *pI* pars intermedia, *ME* median eminence. The hypothalamico-neurohypophysial tract is indicated by three axons ending on capillary vessels of the *NH*. With a square is indicated approximately the region I described in this paper.

microtome. Observations were carried out with an RCA EM2D microscope provided with a Canalco stigmator.

OBSERVATIONS

1. Electron microscopic observations of the neurohypophysis in normal toad

In the diagram of Figure 1 is indicated the general anatomical disposition of the hypothalamic neurohypophyseal system of the toad and the regions from which the material was dissected and fixed for the electron microscope study.

The following description applies to the neurosecretory axons found in toads with a normal water balance and which were fixed either after rapid decapitation or after alcohol anesthesia. The region in which the neurosecretory axons were found correspond: I) to the hypothalamus proper near the preoptic nucleus, II) to hilar regions of the infundibular process, III) to the vicinity of the nerve terminals and IV) at the nerve endings on the capillaries of the neurohypophysis.

I) *Neurosecretion in nerve axons of the hypothalamus*

In electron micrographs of the hypothalamus near the preoptic nucleus axons containing neurosecretory material can be observed (Fig. 2). Unmyelinated nerve fibers between 0.5 to 1 μ or more in diameter appear which contain different types of structures. In some axons smooth contoured neuroprotofibrils of 200–350 Å in diameter are abundant; in others a vesicular material is also observed.

Neurosecretion consists of granules of high electron density in the center and a definite membrane at the periphery. Clusters of these granules are seen intermingled with the dense vesicular material and on the basis of sizes and density intermediary stages between them can be traced. In other axons there is predominance of the vesicles, but a few of them have a denser content of neurosecretion. Measurements on 44 of these granules give a diameter varying between 460 to 920 Å with a mean of 620 Å (Table 1). In these hypothalamic nerve fibers, mitochondria are also present and they can be clearly differentiated from the vesicular material and the granules of neurosecretion.

II) *Neurosecretion in hilar areas of the infundibular process*

In electron micrographs of hilar areas of the supraoptico-hypophyseal tract, situated within the infundibular process proper, pituicytes and axons containing secretory material can be found. In Figure 3 portions of two pituicytes showing the nucleus and several details of the fine structure of the cytoplasm are seen. In the bottom part there are two axons containing neurosecretory granules that are definitely larger than those observed in the hypothalamus. These granules are best seen in Figure 4, within parallel unmyelinated axons. The granules show high electron density and are

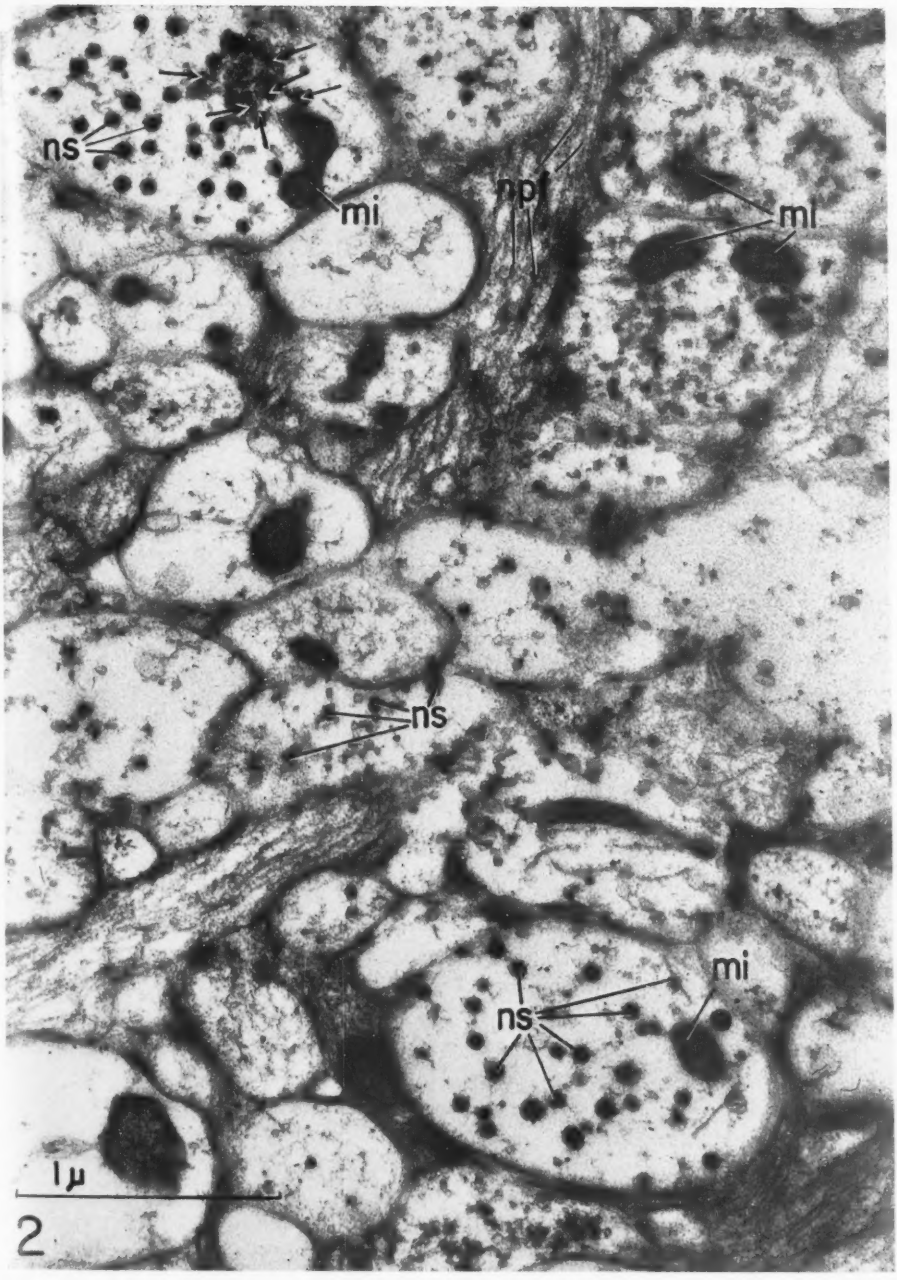

FIG. 2. Electron micrograph of axons of the hypothalamus in region I (see Fig. 1) of a normal toad. Several axons containing neuroprotofibrils (*npf*), mitochondria (*mi*) and neurosecretion (*ns*) are observed. There is a vesicular material within which the *ns* is formed (arrows). 41000×.

practically homogeneous throughout. However, in many of them a thin surface membrane separated from the dense granule by a clear space can be observed. The size of the granules in this hilar region is definitely larger than in the hypothalamus. In a total of 69 measurements the sizes varied between 600 and 2500 Å with a mean of 1350 Å (Table 1). In Figure 4 other details of structure of the nerve fibers can be observed. Some of the axons appear enlarged and with a watery aspect and contain mitochondria, neurosecretion and a few membranous components interpreted as endoplasmic reticulum. In the others the axoplasm is denser and shows abundant neurofibrillar elements of two types. Some are neurofilaments (less than 100 Å thick) and others neuroprotofibrils of 200–300 Å in diameter with smooth contours and tubular aspect similar to those described by

TABLE 1. SIZE OF NEUROSECRETORY GRANULES AND SYNAPTIC VESICLES IN NEUROHYPOPHYSIS

	Region	No. of observations	Range in Å	Mean size in Å
Neurosecretion	I. Axons in hypothalamus	44	460– 920	620
	II. Axons in hilar infundibular region	69	600–2000	1350
	III. Axons near ending	65	1000–2000	1500
	IV. Terminal ending	76	600–1700	1150
Synaptic vesicles	IV. Terminal ending	200	250– 750	430

De Robertis and Franchi (14) in isolated axoplasm. In these denser axons a few neurosecretory granules aligned in rows, mitochondria and some large clear vacuoles can be found.

III) *Neurosecretory material in the nerve axons near the terminal*

Region III, situated near the blood vessels of the infundibular process, correspond to enlarged axons of irregular shape filled with neurosecretory material and where the other components of the axon are less apparent (Fig. 5).

The neurosecretory granules are similar to those described in the hilar axons. There are mitochondria with a bizarre shape, that can be readily differentiated from the granules and only a few neuroprotofibrils and vacuoles. In this region the neurosecretory granules reach the largest size with a mean diameter of 1500 Å in 65 determinations (Table 1).

IV) *Neurosecretory material at the terminal ending*

The terminal portion or ending immediately adjacent to the capillaries is characterized by the presence of two types of neurosecretory material (Figs. 6 and 7). One is represented by the dense neurosecretory granules described above, and the other by a new vesicular component which is

FIG. 3. Electron micrograph of the hiliar region of the neurohypophysis (region II). Two pituicytes are seen and at the bottom some neurosecretory axons. *Pn* pituicyte nucleus. *Ax* axon. 26000×.

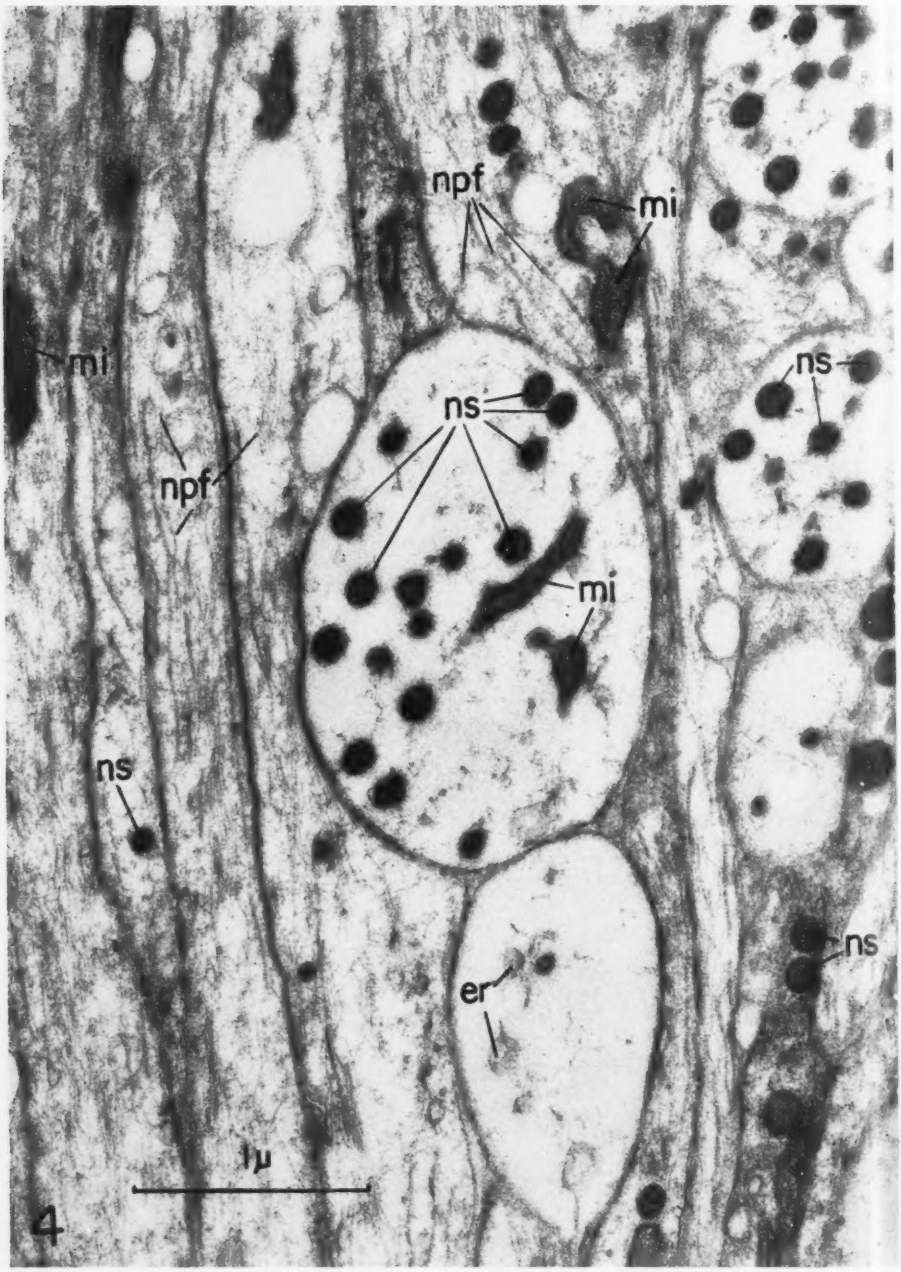

FIG. 4. The same as Fig. 3. Several axons containing neurosecretion (*ns*) are seen. Some of them have swollen segments. The neurofibrillar substance is composed of neuroprotofibrils (*npf*) and finer neurofilaments. Mitochondria (*mi*) are readily differentiated from the granules. Some large clear vacuoles are also seen. 36000×.

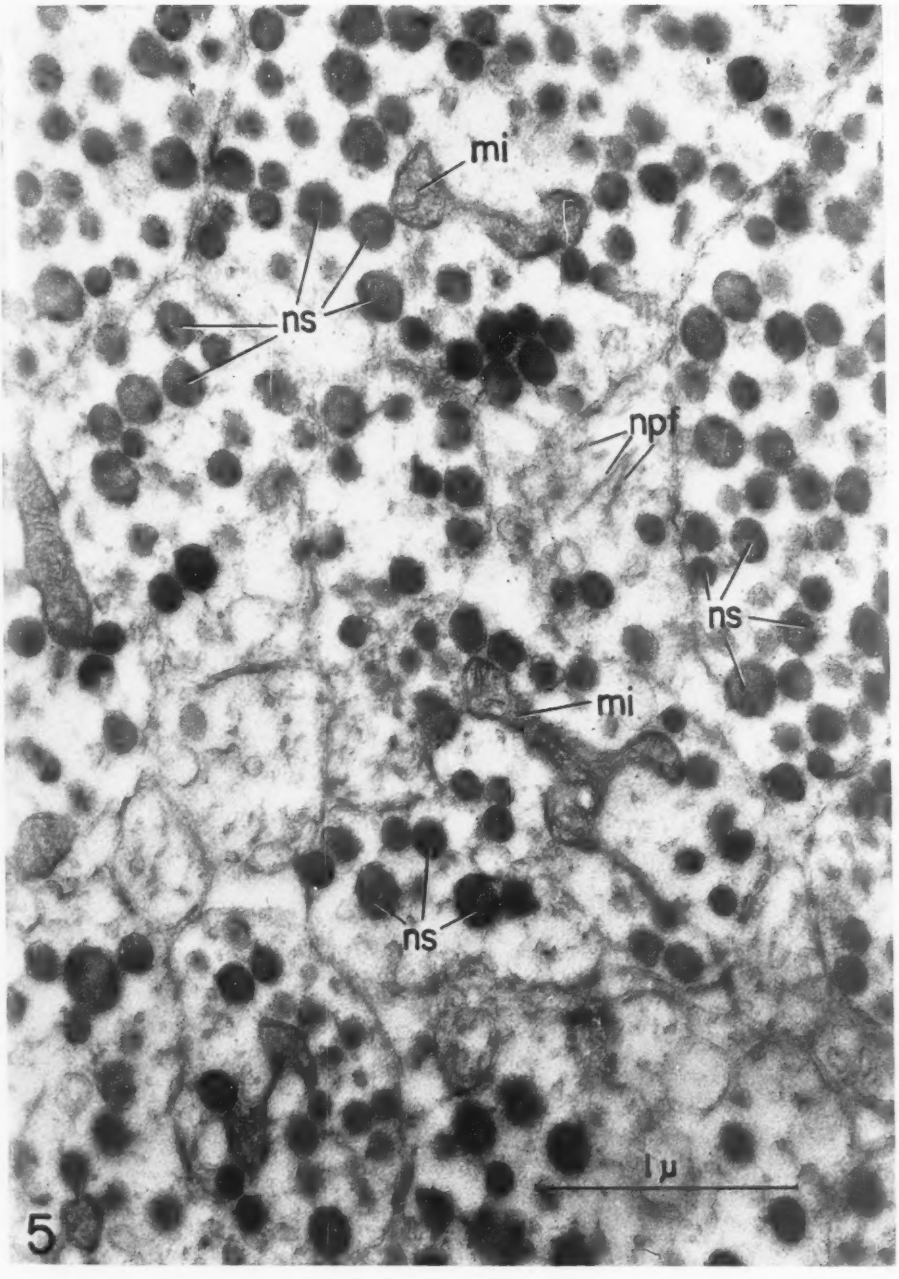

FIG. 5. Electron micrograph of region III (near the ending) of a normal neurohypophysis. The axons are considerably larger and filled with neurosecretion. A few neuroprotofibrils remain and bizarrely shaped mitochondria are present. 40000×.

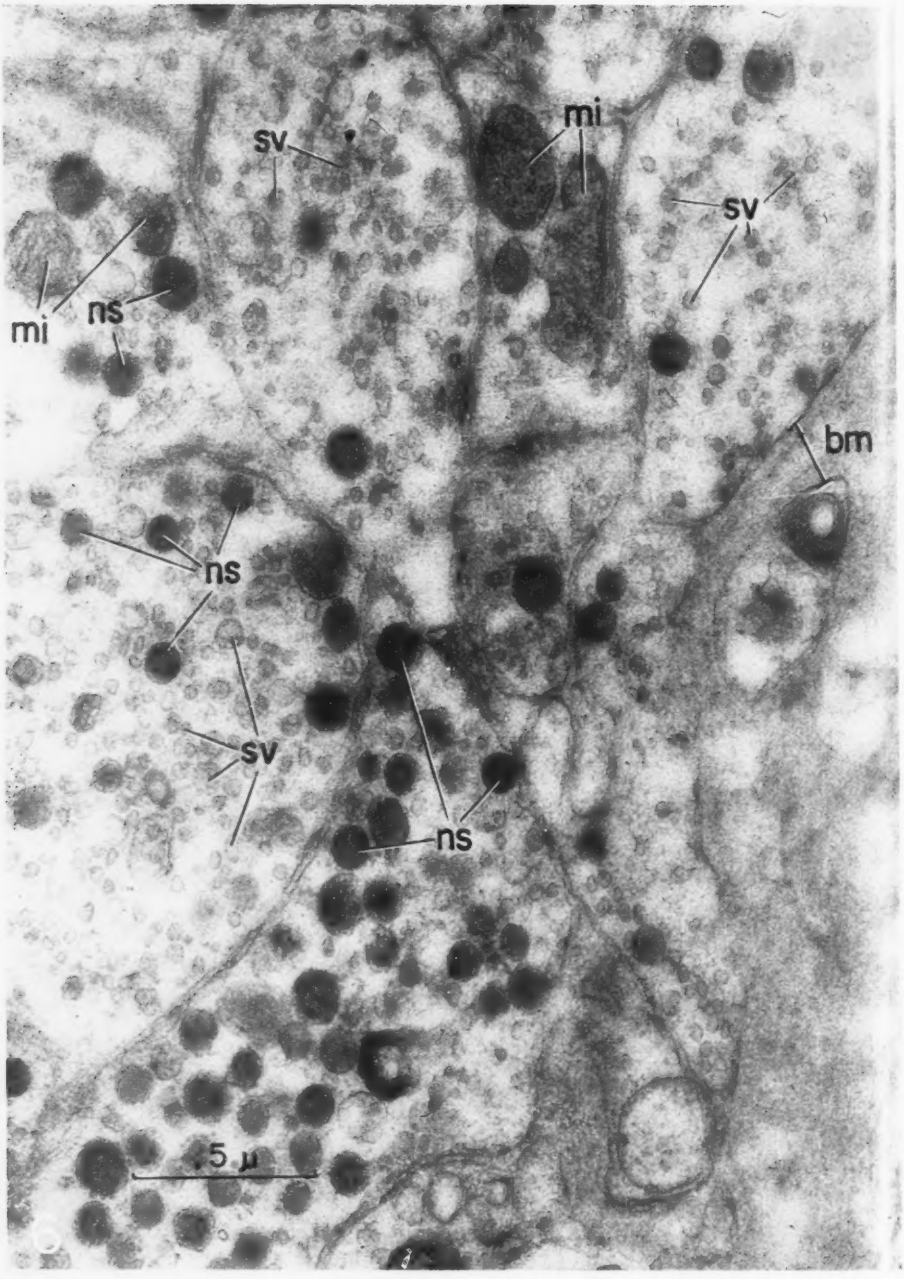

FIG. 6. Electron micrograph of region IV (at the ending) of a normal neurohypophysis. Within the enlarged endings there are neurosecretion granules (*ns*) surrounded by a membrane and synaptic vesicles (*sv*). A thick fibrillar basement membrane is seen near the capillary. 57000×.

similar in size, shape, and density to that found in synaptic terminals and in other nerve endings (15) and which were originally described as synaptic vesicles by De Robertis and Bennett (16). This name will be tentatively applied here to this component.

In this region the density of the neurosecretory granules may vary considerably and some of them, found near the capillary, show a decreased density as if some material had been released from them (Fig. 7). In these normal animals membranes apparently corresponding to empty neurosecretory granules can be found. The size of the granules is in this region slightly smaller, of the order of 1200 to 1100 Å in 76 measurements (Table 1).

The synaptic vesicles of the neurohypophysis are morphologically similar to those found in central and peripheral synapses. They are round or ovoid with a definite membrane 40–50 Å thick and a clear content that has a density slightly higher than the matrix (Figs. 6 and 7). The detailed observation of this vesicular component shows that it has no relationship with the granules of neurosecretion. There are no intermediary forms in between them and the measurements show a completely different range of dimensions. In 200 determinations the mean diameter was of the order of 430 Å with sizes ranging between 250 and 750 Å (Table 1).

In Figure 8 histograms of the diameter of 200 synaptic vesicles and of 180 neurosecretory granules in the axons and endings of regions III and IV are shown. The wider distribution of the granules may be due in part to the fact that many of them may be sectioned at different levels from the equator. The distribution of the two components shows practically no overlap, with peaks that are at a significant distance one from the other.

The observation of a large number of electron micrographs shows that the synaptic vesicles, that are concentrated in the terminal, are mixed in different proportions with the neurosecretory granules in region IV. In some instances neurosecretion or synaptic vesicles are found almost exclusively, but in the majority there is a mixture of the two components in different proportions (Figs. 6, 7 and 13).

In this terminal region large mitochondria can be found, some of which have an arched or more bizarre shape. They can be clearly differentiated from the neurosecretory material and from the synaptic vesicles and there are no intermediary forms between them.

In this region IV other details of the relationship between the endings and the large blood capillaries are apparent. The endings are in direct contact with a thick basement membrane which shows a definite fibrillar structure and beyond this there is a thin dense endothelium containing clear vacuoles. The thickness and development of the basement membrane is really striking. Another interesting feature is the lack of glial expansions around the capillary walls that is regularly found in other parts of the CNS (17).

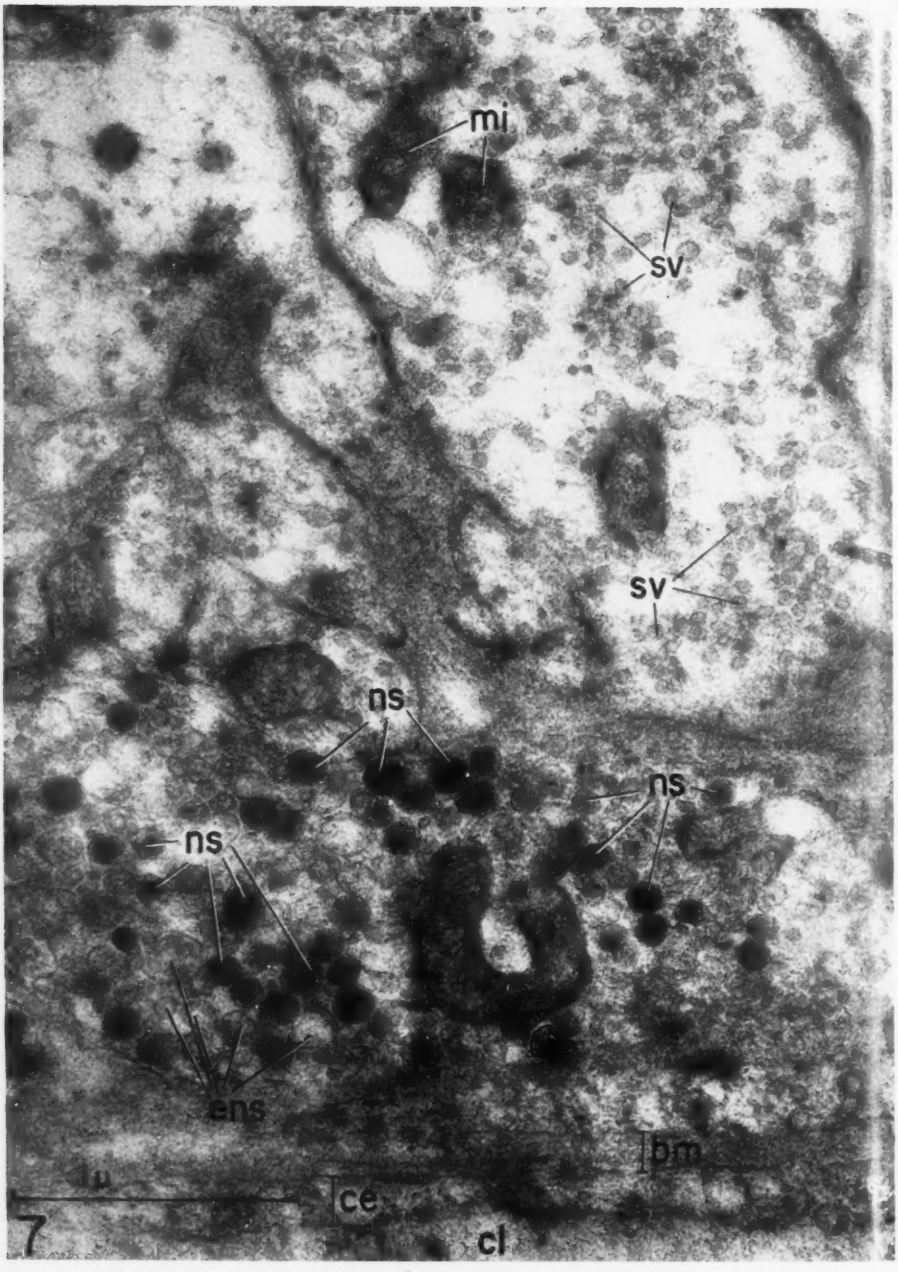

FIG. 7. The same as Fig. 6. The capillary lumen (*cl*), the capillary endothelium (*ce*) and the basement membrane (*bm*) are seen at the bottom. The enlarged endings are filled with *ns* granules some of which appear to have released part of the content. The synaptic vesicles are abundant in some parts of the endings (*sv*). 44000×.

2. Electron microscope observations on the neurohypophysis of experimental animals

The following description corresponds to changes observed in the infundibular portion of the neurohypophysis under experimental conditions known to produce the release of the neurohypophyseal hormones. These changes were more apparent in the region of the ending proper or region IV described above in the normal controls.

a) Neurosecretion in toads after long periods of dehydration

The considerable changes produced by the prolonged dehydration of the animal are illustrated in Figure 9. This is a low power electron micrograph

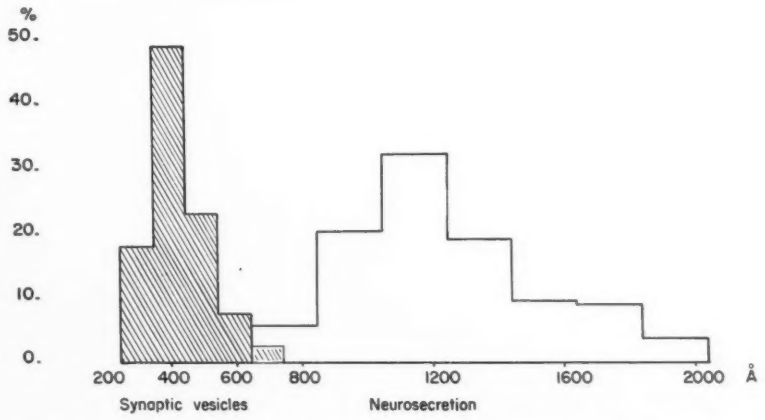

FIG. 8. Histogram showing the percentage distribution of synaptic vesicles and neurosecretory granules in the normal toad.

of one of the large capillaries of the infundibular process with the endothelium and the thick, fibrillar basement membrane that sends expansions in between some of the terminals. The most striking change that has occurred is the disappearance of practically all neurosecretory granules from the endings in region IV. A zone of several micra around the capillary is now practically devoid of granules. In this region the endings are filled only with synaptic vesicles. This is even more clearly seen in Figure 10 at higher magnification. Only a few neurosecretory granules are found at the endings which are filled with numerous, tightly packed synaptic vesicles.

Some of the clusters of synaptic vesicles accumulate near the periphery of the endings and appear to enter into direct relationship with the surface membrane. In region III away from the terminals the axons are filled with neurosecretory material as in the normal.

b) Neurosecretion in toads injected with hypertonic NaCl

The injection of hypertonic NaCl is known to induce a rapid release of antidiuretic hormone from the neurohypophysis. Under the electron micro-

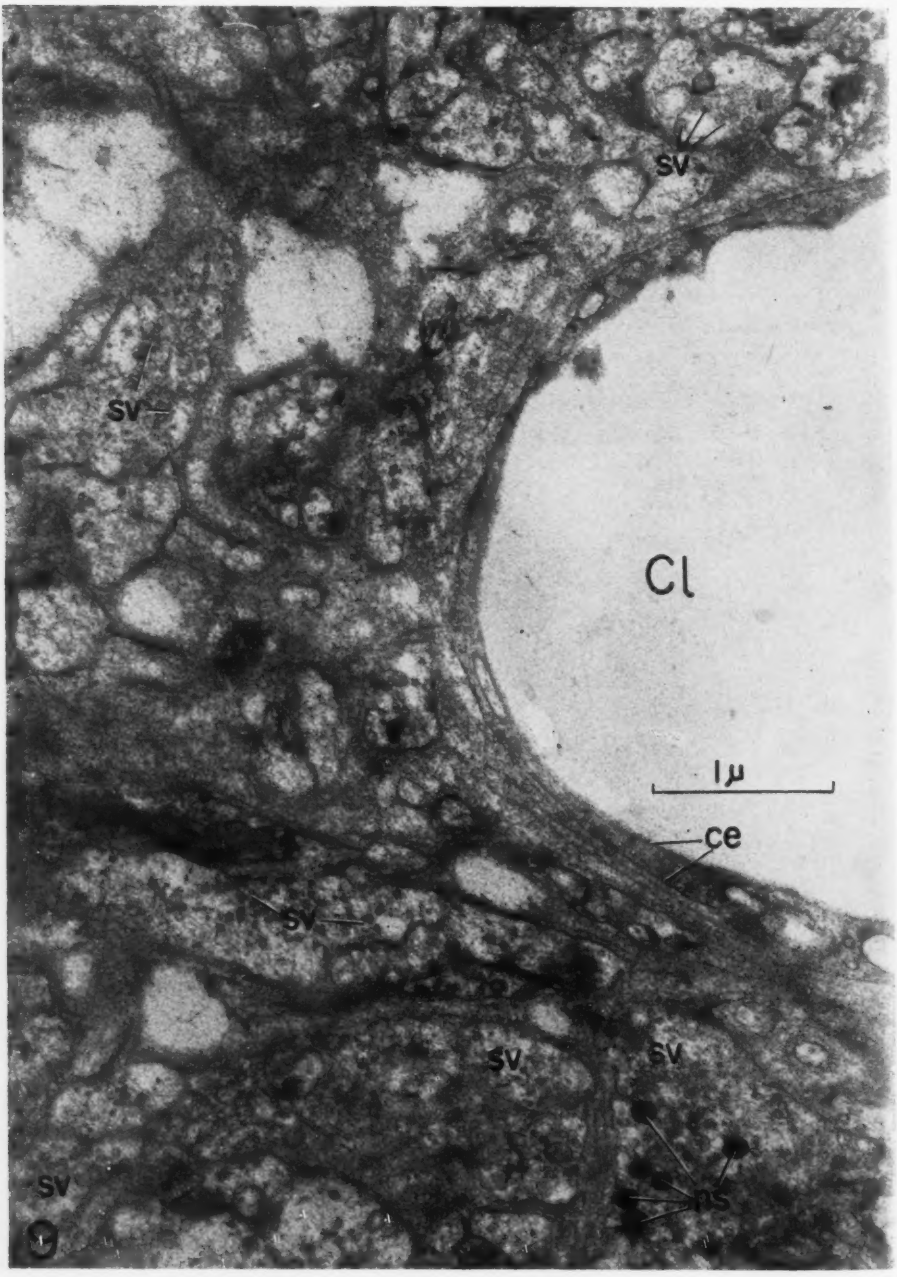

FIG. 9. Electron micrograph of region IV of the neurohypophysis of a toad after chronic dehydration. *cl* large capillary lumen, *ce* capillary endothelium. Numerous endings abutting the basement membrane are observed. They show numerous synaptic vesicles (*sv*), but practically no neurosecretion (*ns*). Compare with Figs. 6 and 7. 29000×.

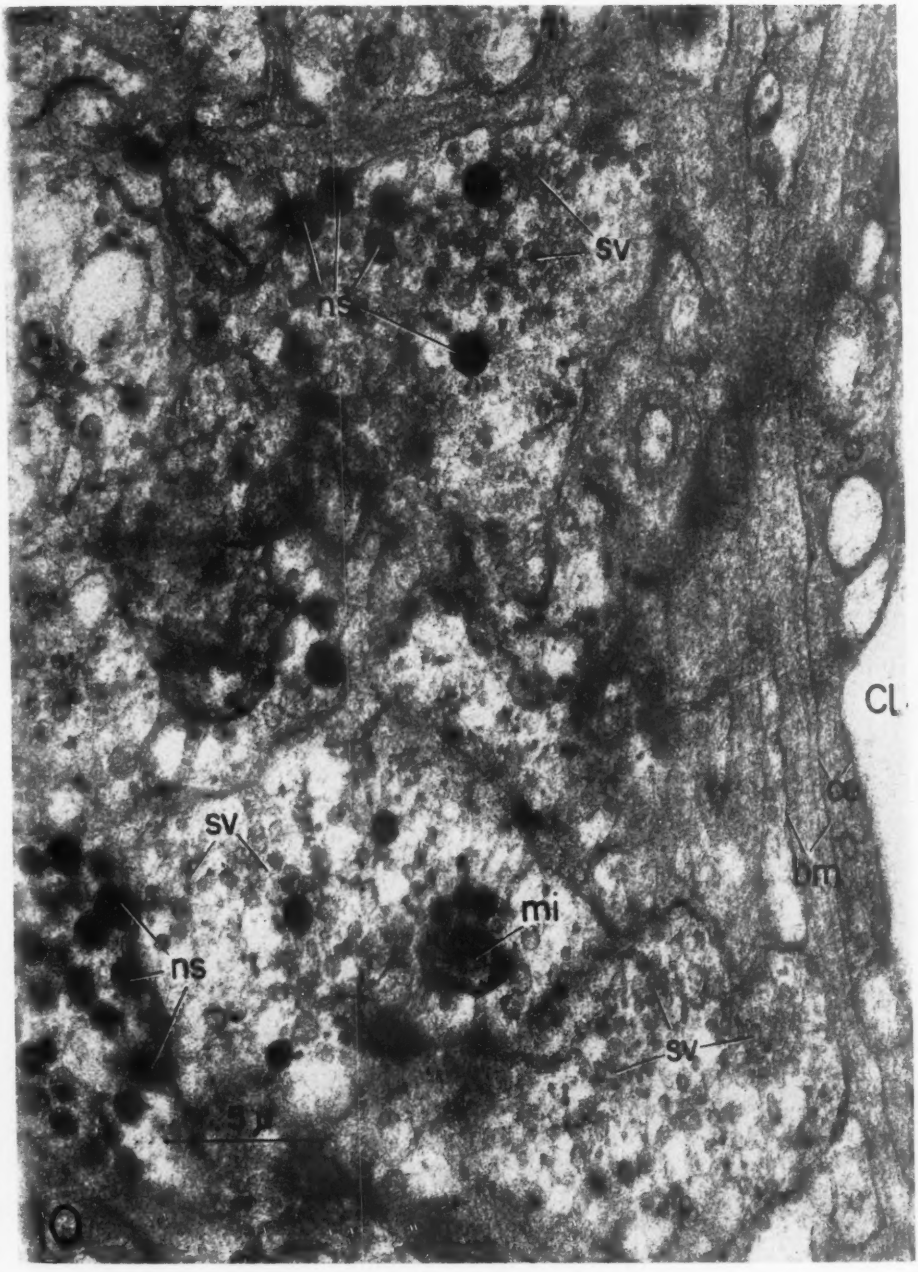

FIG. 10. The same as Fig. 8 at higher magnification. Only few neurosecretory granules are seen in region IV. They are more numerous away from the capillary (region III). The synaptic vesicles seem to have increased in number and they are packed at certain spots near the membrane. 50000×.

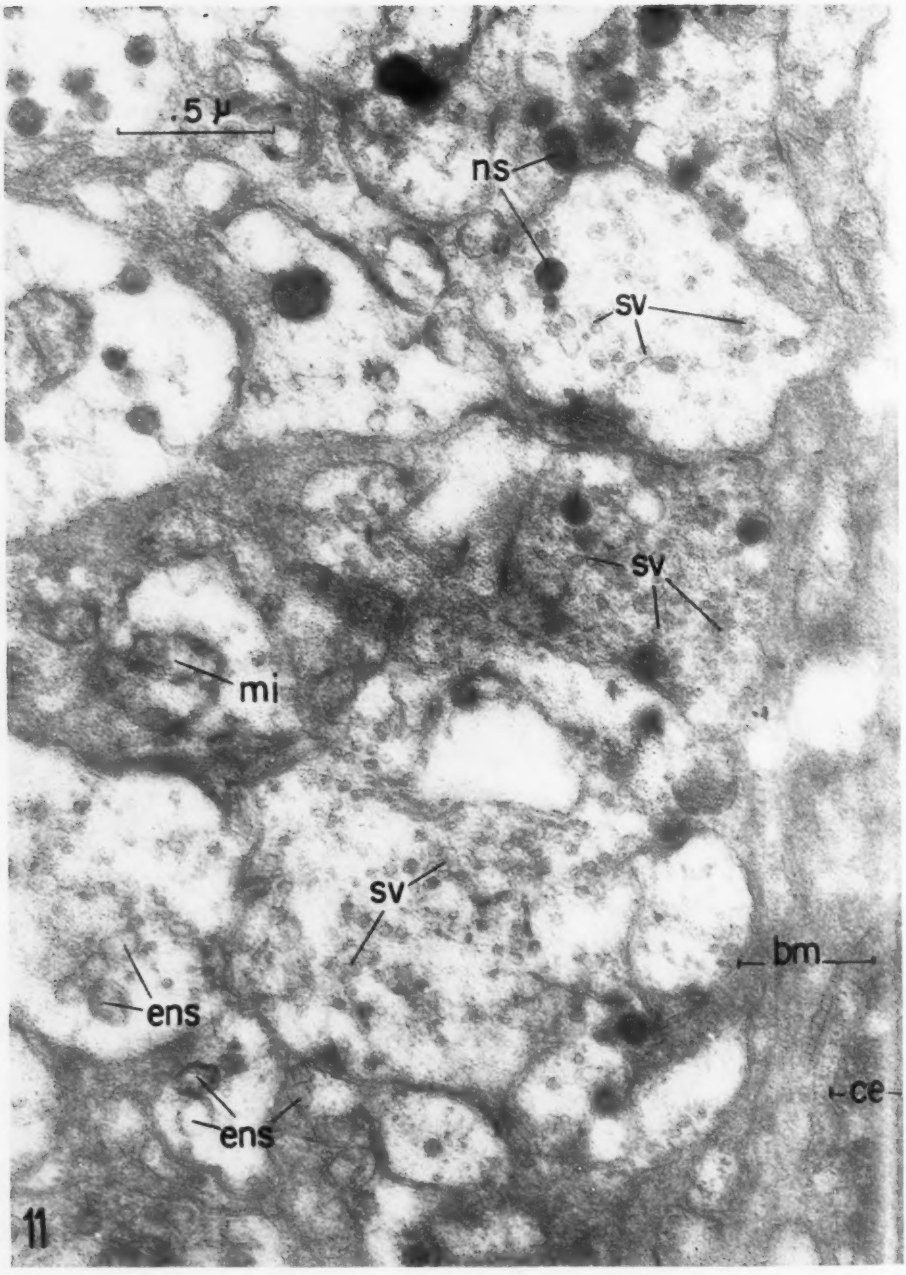

FIG. 11. Electron micrograph of region IV in a toad injected 10 minutes previously with hypertonic NaCl. The endings look very pale and are lacking neurosecretion. A few empty granules (*ens*) are seen. *mi* swollen mitochondria. The synaptic vesicles (*sv*) seem also to have decreased in number and size with respect to the normal (see Fig. 7). 48000×.

scope the pericapillary endings of region IV are practically devoid of neurosecretory granules (Fig. 11). Only a few of them are found and several empty membranes remain that are interpreted as evacuated granules. Another interesting change is the swelling of the mitochondria, which was also found in some endings after prolonged dehydration, but which is more conspicuous after this rapid and massive treatment.

The nerve endings are rather pale and in many cases devoid of synaptic vesicles. Other endings show a number of them but on the whole there is a clear difference from the controls and particularly with the toads after long dehydration.

c) Neurosecretion after ether anesthesia

In toads anesthesized with ether which is known to elicit a massive release of neurohypophyseal hormones, the pericapillary endings are practically devoid of secretory granules (Fig. 12). The entire ending is filled with empty membranes surrounding clear vacuoles of different size. Only a few of the smaller vesicles can be recognized as synaptic elements, the rest is a mass of vacuolated cytoplasm. Mitochondria show no definite changes with this treatment.

DISCUSSION

Origin and evolution of the neurosecretory granules

The observations reported here are in general agreement with the established concepts of the neuronal origin of secretion in the neurohypophysis (see reference 2–3). While in the past it was claimed that parenchymatous cells of the posterior lobe were responsible for the secretion, the findings of cytological changes in the pituicytes (18) were disproven by the independent work of Hickey *et al.* (19) and De Robertis and Primavesi (20).

The electron microscope observations have in general recognized that the neurosecretory granules are found within the axons (5). However the presence of small granules within pituicytes is reported by Green and van Breemen (4) and Green and Maxwell (8). In our material we have clearly observed that the neurosecretion is contained within the axons and nerve endings and that the pituicytes have no such material.

The prevailing concepts favor the view that the neurosecretory material is synthesized within the pericaryon of the hypothalamic nuclei (preoptic nucleus in the toad) and then migrates along the axons to be released at the endings in the infundibular process of the neurohypophysis. Thus the accumulation of neurosecretion above the cut in the neurohypophyseal stalk (21) has been considered as an important evidence for this centrifugal movement of neurosecretion.

Recently, however, this theory of the axoplasmic streaming has been criticised by Green and Maxwell (8) who on the bases of certain calculations reach the conclusion that probably the antidiuretic hormone is formed all along the pathway of the axon. The authors also postulate that this

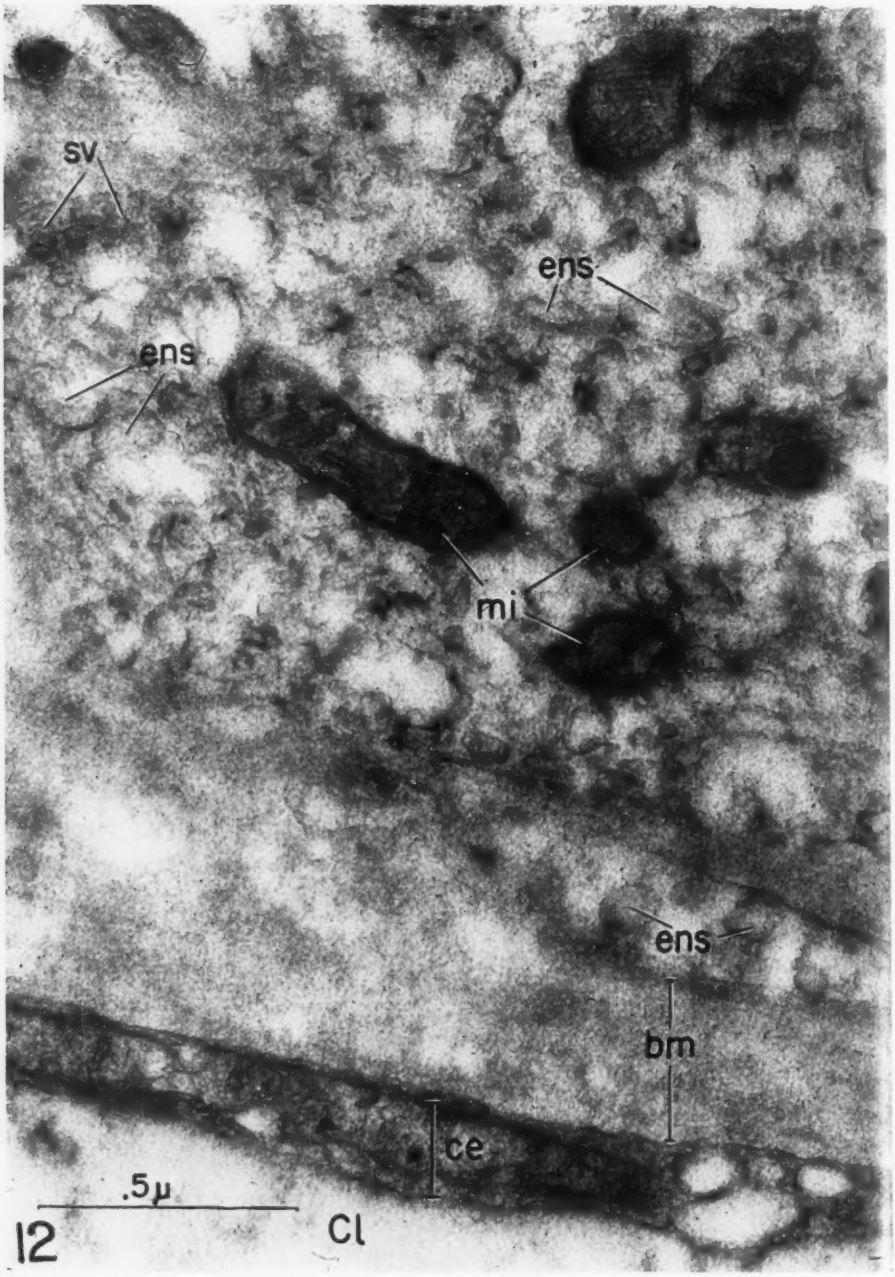

FIG. 12. Electron micrograph of region IV of a toad anesthesized with ether. Practically all neurosecretory granules have disappeared and the cytoplasm looks vacuolated. *mi* mitochondria with normal aspect, *bm* thick basement membrane. 80000×.

might be due to the formation of neurosecretory granules from the mitochondria present in all segments of the axon. Our observations that are summarized in the diagram of Fig. 13A throw some light on these conflicting views. The finding of a definite increase in size of the granules along the preoptic-neurohypophyseal tract seem to us of fundamental importance.

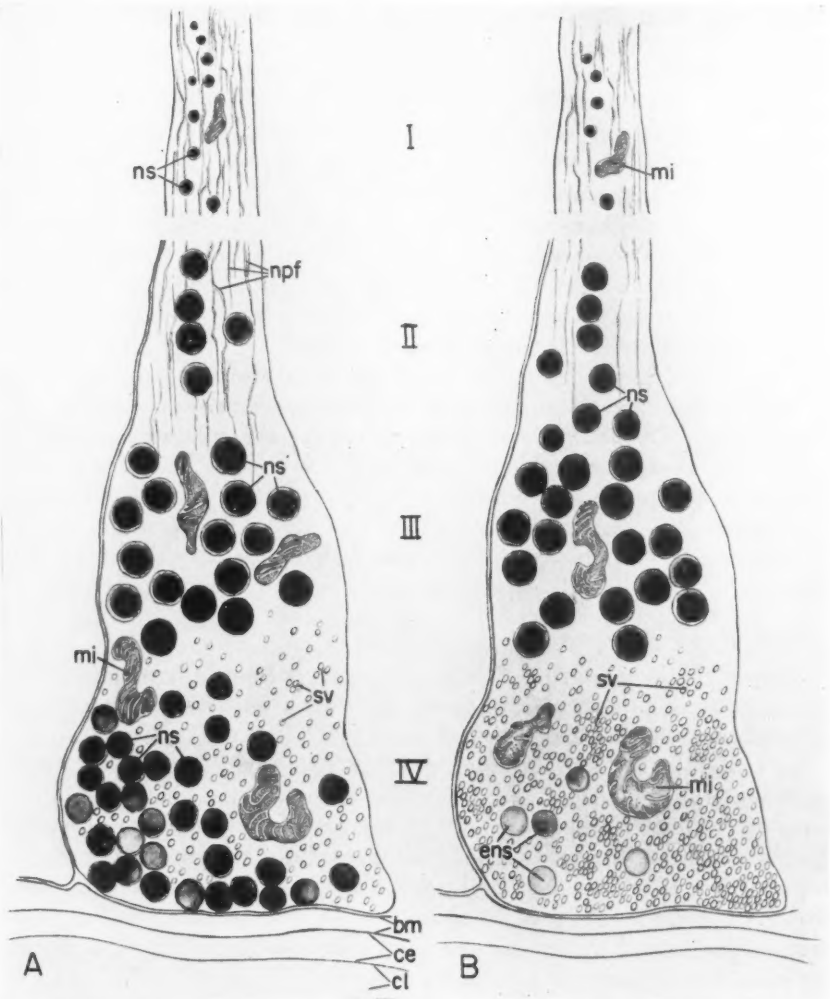

FIG. 13. Diagram of a neurosecretory axon in the different regions (I, II, III and IV) described in the paper. A. From a control animal. B. From a chronically dehydrated toad. See the release of neurosecretion (*ns*) from region IV and the increase in synaptic vesicles (*sv*). *npf* neuroprotofibrils. *mi* mitochondria. The relative size of the granules in the different regions and of the synaptic vesicles is maintained. *bm* basement membrane, *ce* capillary endothelium, *cl* capillary lumen.

As indicated in Table 1 the neurosecretory granules increase from 620 Å in region I of the hypothalamus to 1350 Å and 1500 Å in the infundibular process. Finally at the terminal the mean diameter appears to be reduced (1150 Å) probably because of the liberation of the secretion. These changes in dimensions of the granules are interpreted as indicating that the synthesis of neurosecretory material, probably initiated in the pericaryon, continues along the axon so that the secretion reaches complete maturation near the ending. In view of our findings the concept of axoplasmic streaming of the secretion should be supplemented with that of a *progressive synthesis along the axon*. This combined concept may probably explain the difficulties found by Green and Maxwell (8) to calculate the rate of formation and streaming of the granules considering the pericarya of the hypothalamic nucleus as the only source of synthesis.

Our observations clearly show that the neurosecretory material is formed within a *dense vesicular component* of the axon and *not from mitochondria* as assumed by Green and Maxwell (8). These vesicles become filled with a dense homogeneous content and even the largest granules show the presence of a single enveloping membrane.

The high electron density of the granules is probably indicative of a dense molecular packing within the membrane. The presence of —SH and S—S-groups demonstrated histochemically in the neurosecretion of the toad (22) may also explain the high density by a strong reduction of the osmium tetroxide. The presence of phospholipids may also contribute to the electron opacity. Thus Baker's test for phosphatids was found positive in neurosecretion (23) and lecithinases were found to remove the neurosecretion from a histological section of the toad's neurohypophysis (24).

Release of neurosecretion

It is generally admitted that the neurohypophyseal hormones are secreted continuously in a steady state which can be changed more or less abruptly by different experimental conditions.

In control animals anesthesized with alcohol—which is known not to induce release of the hormones (13)—we have observed that in the nerve endings (region IV) the granules not only are slightly smaller but show a variable electron density (Fig. 7). This is at variance with the granules of region III in which the density is practically similar. This finding, complemented by the presence of large vesicles formed by the membranes of emptied granules, is interpreted as morphological evidence of this continuous release of secretion at the ending.

The process of release is greatly increased in animals in which there is a massive output of hormones as in the case of ether anesthesia (12) or after the injection of hypertonic NaCl. Also after chronic dehydration the release of neurosecretory granules from the ending is almost complete. The finding of numerous empty vesicles and membranes in the acute experiments is indicative that the release of the granules is by liberation of the small molecular components contained within the membrane and not by

the discharge of the entire granule. In this sense the mechanism seems to be to some extent similar to that found by De Robertis and Vaz Ferreyra (25) in the release of the catechol containing droplets from the adrenal medulla after stimulation of the splanchnic nerve.

The finding that the release affects mainly region IV (ending) while the content of granules is maintained or may be even increased in the other regions (Fig. 13B) is probably related to other important properties of the endings.

Significance of the synaptic vesicles in neurohypophysis

The observations of a small vesicular component that has the morphological characteristics of the synaptic vesicles described by De Robertis and Bennett (16) in many synapses (see 15) is of particular interest. Palay (5) who found this small vesicular component in the endings of the neurohypophysis interpreted them as synaptic vesicles. Green and Maxwell (8) have also recognized a pale vesicular component but their interpretation is not very definite. For these authors there is probably a gradation between the dense and the pale granules which might be related to the physiological state of the gland.

The findings reported here definitely show that the neurosecretion granules and the synaptic vesicles are two essentially different components. While the granules are found all along the preoptic-neurohypophyseal tract the synaptic vesicles are confined to the ending (Fig. 13A). Furthermore the differences in size (Fig. 8) and morphological and experimental behavior of both components leave no doubt about their special identification.

The synaptic vesicles apparently do not follow the changes observed in the secretion granules. In chronic dehydration there is a release of the granules but the vesicles are maintained and they may even increase in number. Although actual measurements were not performed the impression is gained that the packing density of the synaptic vesicles has increased with this treatment (Fig. 10). This fact is reminiscent of the increase in the number of synaptic vesicles found in the nerve terminals of the adrenal medulla after stimulation of the splanchnic nerve with impulses of a certain frequency (26). It is now known that in the neurons of the hypothalamic nuclei (27) the rate of firing is changed by the injection of hypertonic solutions (27). This finding seems to us of considerable importance in explaining the release of the secretion at the ending and the changes in the synaptic vesicles. From all known data of the literature (15) it seems now possible that the synaptic vesicles contain acetylcholine or other chemical mediators and that these are released under the influence of the nerve impulse. Furthermore the experiments of De Robertis and Vaz Ferreyra (26) have shown that a balance may exist between the rate of formation of the vesicles at the endings and the release by stimulation so that at certain frequencies the number of vesicles increases while at others they may be depleted (15).

The hypothesis is put forward here that in the nerve endings of the neurohypophysis the synaptic vesicles may change under the action potentials delivered by the neurons and arriving at the ending.

The nerve impulses may elicit the release of a mediator from the ending and at the same time they may change the rate of formation of the vesicles so that in some cases (chronic dehydration) they may increase in number, or in others they may be depleted (acute dehydration, ether). This working hypothesis is extended further by postulating that the synaptic vesicles may in some way elicit the actual release of the neurosecretion from the ending. According to this hypothesis the synaptic vesicles would have a role similar to that of the nerve endings of the adrenal medulla whose stimulation produces the secretion of acetylcholine and this in turn the release of the catechol amines from the adrenal cell. In this case, however, both neurohumoral mechanisms would be integrated within the same cell.

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EFFECTS OF MODERATE DECOMPRESSION STRESS UPON PLASMA POTASSIUM AND GLUCOSE IN THE ADRENALECTOMIZED CAT[1]

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ABSTRACT

Under conditions of moderate decompression stress (exposure to a simulated altitude of 28,000 feet for 60 minutes), intact cats showed a decrease in plasma potassium concentration from 4.42 m.eq./L. to 3.04 m.eq./L. and an increase in plasma glucose from an initial value of 94 mg.% to 354 mg.%. Chronically adrenalectomized cats, maintained on cortisone and DCA, exhibited, under similar conditions, a reduction in plasma potassium from 4.85 m.eq./L. to 3.65 m.eq./L. and a rise in plasma glucose from 103 mg.% to 286 mg.%. No significant difference was found between the plasma potassium or glucose responses of the two groups. Cats in moderate adrenal insufficiency demonstrated similar changes, plasma potassium decreasing from 6.04 m.eq./L. to 4.84 m.eq./L. and plasma glucose rising from 101 mg.% to 272 mg.%. On exposure of adrenalectomized cats maintained on supportive therapy to conditions of severe decompression stress (40–45,000 feet), however, plasma potassium concentration rose markedly to almost twice the initial value, while the plasma glucose increased three- to fourfold.

The results agree with those obtained in similar experiments conducted on dogs, and tend to contradict previous results involving anesthetized, acutely adrenalectomized cats.

IN A recent report from this laboratory (1) it was shown that in dogs the hypokalemic response to moderate decompression stress is not appreciably altered by removal of the adrenal glands. Previous experiments with cats (2) and rats (3) indicated that in these animals, on the other hand, the response is abolished by adrenalectomy, and is therefore apparently mediated by the adrenal glands.

In reviewing the procedure used on the cat (2), it was noted that anesthetized, acutely adrenalectomized animals[3] were used, the experiments

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[3] In this paper the term "chronically adrenalectomized" refers to animals which were allowed a postoperative recovery period of at least two weeks before being subjected to the experimental procedure. "Acutely adrenalectomized" refers to animals subjected to experimentation within two hours after operation.

being conducted within two hours following the operation. Experience with dogs (1) has indicated that anesthesia, combined with postoperative trauma, tends to diminish, and sometimes abolish, the potassium response to an altitude of 30,000 feet.

The principal objective of the present study was to determine the plasma potassium response of the unanesthetized, chronically adrenalectomized cat[3] to moderate decompression stress. The response of plasma glucose to the same conditions was also measured.

METHODS

Three male and eight female cats, weighing between 1.5 and 2.5 kg., were subjected to decompression stress in the intact state. Five of the animals were then adrenalectomized bilaterally via midline abdominal incision, and subsequently maintained on daily injections of cortisone acetate (5 mg. per day, i.m.) and desoxycorticosterone acetate (0.5 mg. per day, i.m.). Except in five instances, to be described, injections were continued through the day prior to an experiment.

The experimental procedure was identical on both intact and adrenalectomized animals. A blood sample (5 cc.) was taken from the left heart by cardiac puncture. Immediately thereafter the animal was placed, unrestrained, in an altitude chamber and decompressed to a simulated altitude of 28,000 feet within 2 to 3 minutes. At the end of 60 minutes of exposure to this altitude, the animal was returned to ground level, removed from the chamber, and another blood sample taken by cardiac puncture within one minute. Control experiments were carried out in an identical manner, except that actual decompression was not accomplished. A period of at least two weeks was allowed to elapse between repetitive experiments on any animal, in order to permit complete recovery from the procedure.

In five instances, adrenalectomized cats were allowed to become moderately insufficient by withholding cortisone and DCA for the four days preceding the experiment.

Plasma potassium concentrations were determined by internal standard flame photometry, and plasma glucose concentrations by the Somogyi method (4) using Nelson's arsenate molybdate reagent (5).

RESULTS

Effects of moderate decompression stress on intact cats

Table 1 indicates the effects of decompression stress on plasma potassium and glucose concentrations in ten experiments carried out on intact cats exposed to a simulated altitude of 28,000 feet for 60 minutes. Nine different animals were used in this series. In every case there was a decrease in plasma potassium after exposure. The change in the mean value from 4.42 m.eq./L. to 3.04 m.eq./L. represents a decrease of 31.2%. Blood glucose concentration, on the other hand, was found to be increased in each case after decompression, with the mean value rising from 94 mg.% to 354 mg.%, an increase of 277%. In nine control experiments, summarized at the bottom of the table, no significant change was observed in either plasma potassium or glucose concentration.

TABLE 1. EFFECTS OF MODERATE DECOMPRESSION STRESS ON INTACT CATS*

Cat No.	Expt. No.	Predecompression		Decompression	
		Plasma K (m.eq./L.)	Plasma glucose (mg.%)	Plasma K (m.eq./L.)	Plasma glucose (mg.%)
4	23	3.79	86	2.27	183
5	31	4.06	74	3.43	485
10	34	4.37	88	2.43	505
15	17	4.25	94	3.05	244
17	15	5.14	86	3.46	331
17	30	3.84	94	2.68	369
18	35	4.23	91	2.77	313
19	16	5.66	127	4.55	279
20	18	4.85	98	3.40	283
21	50	4.03	100	2.34	552
Mean		4.42	94	3.04	354
S. E.†		± .19	±4	± .22	±39
Undecompressed Controls					
Mean (9 expts.)		4.57	97	4.40	110
S. E.†		± .17	±6	± .19	±6

* 28,000 feet for 60 minutes.
† Standard error of the mean.

Effects of moderate decompression stress on adrenalectomized cats maintained on cortisone and DCA

As shown in Table 2, a decrease in plasma potassium concentration occurred in each of ten experiments involving four adrenalectomized cats maintained on cortisone and DCA. The change in the mean value from 4.85 m.eq./L. to 3.65 m.eq./L. represents a reduction of 24.7%. As in intact cats, a marked increase was noted in the blood glucose level following decompression. From an initial mean value of 103 mg.% the concentration increased to 286 mg. %, a change of 178%. A summary of nine control experiments, involving adrenalectomized animals, is shown at the bottom of the table. No significant changes are indicated.

Effects of moderate decompression stress on cats in adrenal insufficiency

As a supplement to the preceding studies, five additional experiments were carried out on three adrenalectomized cats from which cortisone and DCA had been withheld to produce moderate adrenal insufficiency. Table 3 shows the results of these experiments. As in intact animals, and adrenalectomized animals maintained on supportive therapy, there is a decrease in the plasma potassium level, and an increase in the glucose concentration.

Effects of severe decompression stress on adrenalectomized cats

Four experiments were conducted on two of the adrenalectomized animals to determine the changes in plasma potassium and glucose under

TABLE 2. EFFECTS OF MODERATE DECOMPRESSION STRESS ON ADRENALECTOMIZED CATS MAINTAINED ON CORTISONE AND DCA*

Cat No.	Expt. No.	Predecompression		Decompression	
		Plasma K (m.eq./L.)	Plasma glucose (mg.%)	Plasma K (m.eq./L.)	Plasma glucose (mg.%)
4	36	4.92	101	3.16	397
	48	4.90	101	3.05	320
	62	4.85	97	3.07	417
	63	4.84	111	3.84	348
5	49	5.00	92	4.39	416
15	26	4.92	140	4.07	142
	39	5.15	108	3.89	198
	52	4.84	97	3.72	280
16	27	4.61	102	3.81	207
	43	4.47	77	3.51	132
Mean		4.85	103	3.65	286
S. E.†		±.06	±5	+.14	±35
Undecompressed Controls					
Mean (9 expts.)		4.66	96	4.76	97
S. E.†		±.33	±5	±.34	±4

* 28,000 feet for 60 minutes.
† Standard error of the mean.

conditions of severe decompression to the point of respiratory collapse, using the criterion (i.e. cessation of respiration for 15 seconds) defined by Kline (2). As shown in Table 4, the mean potassium level rose 3.2 m.eq./L. or 71.6%, while plasma glucose increased 346 mg.% or 323%.

DISCUSSION

The present study shows that adrenalectomized cats tolerate the stress of exposure to various altitudes remarkably well when maintained on daily doses of cortisone and DCA, or when in a state of moderate adrenal

TABLE 3. EFFECTS OF MODERATE DECOMPRESSION STRESS* ON ADRENALECTOMIZED CATS IN MODERATE INSUFFICIENCY†

Cat No.	Expt. No.	Predecompression		Decompression	
		Plasma K (m.eq./L.)	Plasma glucose (mg.%)	Plasma K (m.eq./L.)	Plasma glucose (mg.%)
4	56	6.75	96	4.49	248
	60	6.24	94	4.29	257
15	64	5.82	128	4.37	297
16	57	4.11	100	4.44	182
	59	7.26	88	6.63	377
Mean		6.04	101	4.84	272

* 28,000 feet for 60 minutes.
† Supportive therapy withheld for 4 days preceding experiment.

TABLE 4. EFFECTS OF SEVERE DECOMPRESSION STRESS ON ADRENALECTOMIZED CATS

Cat No.	Expt. No.	Altitude (feet)	Predecompression		Decompression	
			Plasma K (m.eq./L.)	Plasma glucose (mg.%)	Plasma K (m.eq./L.)	Plasma glucose (mg.%)
4	65	40,000	4.50	102	7.81	329
	68	45,000	4.18	100	7.53	309
15	66	45,000	4.69	121	7.96	688
	67	44,000	4.50	106	7.36	484
Mean			4.47	107	7.67	453

insufficiency. No ill effects were found either during or following the procedure. These results are comparable to those obtained using chronically adrenalectomized dogs (1) as well as adrenalectomized rats maintained upon 1% NaCl (3), and suggest that the brief survival reported by Kline (2) of cats subjected to decompression stress 24 hours following adrenalectomy might be a reflection of the influence of recent trauma.

The data in this report indicate that when allowed sufficient time to recover from trauma of operation, the adrenalectomized cat maintained on cortisone and DCA shows the characteristic drop in plasma potassium seen in intact cats, dogs (6), and rats (3, 7) following moderate decompression stress. A similar decrease in cats from which supportive therapy has been withheld would suggest that the effect does not depend upon circulating cortisone or DCA. Furthermore, the sharp increase in plasma potassium shown by two adrenalectomized cats on exposure to severe decompression stress at altitudes of 40–45,000 feet is entirely in accord with results on intact cats (2) and rats (3) under similar conditions.

Kline (2) previously found that acutely adrenalectomized cats subjected to an altitude of 28,000 feet for 90 minutes under anesthesia failed to show a drop in plasma potassium, while Ferguson *et al.* (1) found that dogs, chronically adrenalectomized and unanesthetized, responded to such decompression stress in a manner similar to intact dogs. Pratt *et al.* (3) determined, however, that in contrast to the intact rat, the unanesthetized, adrenalectomized rat showed no fall in plasma potassium after 30 minutes at 25,000 feet. The results of the present study tend to support the view (1) that anesthesia and trauma of recent operation played a large part in the results of earlier work on the cat.

The rise in plasma glucose observed in both intact and adrenalectomized cats following decompression stress is in agreement with results of similar experiments on dogs (8). This would appear to minimize the role of the adrenal gland as a mediator of glucose mobilization and/or utilization during decompression stress. These results, however, are at variance with other studies of glucose levels under various stresses. Ziegler (9) found that intact dogs became hyperglycemic breathing an atmosphere con-

taining 5% O_2 and 95% N_2, or one containing 15% CO_2, 20% O_2, and 65% N_2, while adrenalectomized dogs showed marked hypoglycemia and greatly reduced hyperglycemia respectively in the same atmospheres. Feldman *et al.* (10) found in experiments on rabbits and rats that under conditions of acute anoxia, denervation or removal of the adrenals resulted in hypoglycemia in place of the hyperglycemia noted in intact animals under similar conditions. Prior experimentation with cats involving nerve stimulation (11), emotional stimulation (12), and asphyxia (13) has shown that adrenalectomy results in reduction or abolishment of the hyperglycemia found in intact animals under the same conditions of stress.

In the present study, no significant difference was found between the glucose responses of intact and adrenalectomized cats, although the actual mean rise in the adrenalectomized cat was only 183 mg.% compared to 260 mg.% in the intact cat. The lack of statistical significance might be attributed to large individual variances among the cats in response to stress. Griffith (11) was impressed early with this individual variation, and advised caution in generalization concerning mean glucose levels.

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CORTICOSTEROID ACTIVITY OF A TRANSPLANTABLE ADRENOCORTICAL CARCINOMA IN OSBORNE-MENDEL RATS

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ABSTRACT

An adrenocortical carcinoma, arising in an Osborne-Mendel rat, was tested by a standardized stress procedure, for its ability to secrete mineralocorticoids. The tumor was grown in adrenalectomized male rats and then stress was induced by intraperitoneal injection of 5 ml. of KCl solution per 100 gm. body weight. The dosage of KCl was regulated by changing the molality of the solution. This tumor offered complete protection to rats against 0.16 m. KCl, a dose lethal to comparable rats without tumor. This gives qualitative proof of corticoid secretion by the tumor.

REPORTS on functional activity of adrenocortical tumors of rats have been made by Cohen *et al.* (1), Iglesias and Mardones (2), Houssay *et al.* (3), and Snell and Stewart (4). The transplantable adrenocortical adenocarcinoma under study was one of the 4 observed in a group of female Osborne-Mendel rats fed low protein, low riboflavin, semisynthetic diet, containing 0.06% *p*-dimethylaminoazobenzene (5). This paper reports on the functional activity of this tumor as determined by a standardized stress procedure of potassium intoxication.

MATERIALS AND METHODS

Bilateral adrenalectomy was performed on male, Osborne-Mendel rats through a paravertebral incision. Anesthesia was produced by pentobarbital given intraperitoneally, supplemented with ether inhalation. The rats were about two months old and weighed 200 to 250 gm. at the time of operation. One month later tissue from the adrenocortical carcinoma was transplatned into half of the rats; the others served as controls. These operated rats received a 0.9% saline in place of drinking water. When the transplants grew and were visible as subcutaneous nodules, the saline was replaced with tap water. The carcinoma was also transplanted into intact male rats about 3 months old. All rats were fed a diet of Purina chow pellets.

Stress was produced by the intraperitoneal injection of potassium chloride (KCl) solution at 5 ml./100 gm. body weight, to rats fasted over night (18 hours). The dose of KCl was regulated by changing the molar concentration of the KCl solution used for injections. The dose is expressed as moles (m) of KCl per liter of solution. When the transplanted tumors were 2 cm. or more in diameter, stress was induced with various doses of KCl in adrenalectomized and intact tumor-bearing rats, and at the same time in comparable controls. Some rats were subjected to the stress treatment after removal of the tumors. The time interval between adrenalectomy and induction of stress was

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the same for tumor-bearing rats and their comparable controls. All animals were observed for toxic effects for two hours after the KCl stress, and survivors were saved for at least 24 hours before they were killed. After each stress experiment, the rat was rested for at least one week before it was used for another stress experiment. After removal of a tumor at least a week was allowed for recovery before that rat was subjected to KCl stress. All rats were autopsied at the end of the experiments. The perirenal fat was examined for accessory adrenal bodies, and all organs examined for tumor metastases and other lesions. Tumors, adrenal glands, and accessory adrenal bodies were weighed and specimens of these tissues were taken for histologic examination.

RESULTS

The response of the intact and the adrenalectomized rats to various doses of KCl is listed in Table 1. As these stress experiments were performed 3 to 4 months after adrenalectomy; congenital, accessory adrenal bodies in some of the adrenalectomized rats had developed into macro-

TABLE 1. EFFECT OF VARIOUS DOSES OF KCl, INJECTED INTRAPERITONEALLY, AT THE RATE OF 5 ML./100 GM. BODY WEIGHT, IN INTACT AND ADRENALECTOMIZED MALE OSBORNE-MENDEL RATS, FASTED OVER-NIGHT FOR 18 HOURS

No. of rats	Treatment	KCl molality	Millimoles of K administered per 100 gm. body wt.	% mortality
15	None	0.20	1	100
10	None	0.18	0.9	40
20	None	0.16	0.8	0
8	Adrenalectomized	0.16	0.8	100
6	Adrenalectomized	0.14	0.7	100
11	Adrenalectomized	0.10	0.5	91
12	Adrenalectomized, accessory adrenal bodies	0.16	0.8	100
14	Adrenalectomized, accessory adrenal bodies	0.10	0.5	86

scopic glands a millimeter or more in diameter, at the time the stress was applied. In 3 cases they weighed about 30 mg. each. The response of the rats with accessory adrenal bodies to KCl stress was similar to that of comparable rats without these bodies.

For intact Osborne-Mendel rats the minimum lethal dose of KCl was 0.2m and the maximum tolerated dose was 0.16m. For adrenalectomized rats these values were 0.14m and less than 0.1m respectively. Two rats with unilateral adrenalectomy tolerated a KCl dose of 0.16m.

The response of the tumor-bearing rats, adrenalectomized and intact, to KCl stress is listed in Table 2. Adrenalectomized rats with tumor growth survived the maximum dose of KCl (0.16m) tolerated by intact control rats. The same dose of KCl, a week after the removal of the tumor, was lethal to these animals. A dose of 0.2 or 0.18m KCl was not tolerated by any of the tumor-bearing rats, irrespective of the size of the tumor. The smallest tumor weighed 3.5 gm. and the largest one was 40 gm. The response of the intact rats with transplant growth to KCl stress was similar to those of the comparable adrenalectomized rats. These rats also did not

TABLE 2. EFFECT OF VARIOUS DOSES OF KCl ON MALE TUMOR-BEARING RATS (INTACT AND ADRENALECTOMIZED) BEFORE AND AFTER THE REMOVAL OF THE TUMOR

No. of rats	Treatment	KCl molality	Millimoles of K administered per 100 gm. body wt.	% mortality
15	Adrenalectomized	0.16	0.8	0
12	Adrenalectomized	0.20	1.0	100
8	Adrenalectomized	0.18	0.9	100
3	Adrenalectomized, tumor removed	0.16	0.8	100
10	Adrenalectomized, accessory adrenals	0.16	0.8	0
2	Adrenalectomized, accessory adrenals, tumor removed	0.16	0.8	100
10	Intact	0.16	0.8	0
5	Intact	0.18	0.9	100
2	Intact, tumor removed	0.16	0.8	100

tolerate the maximum tolerated dose of KCl (0.16m) after removal of the tumor. Adrenal glands of the tumor-bearing rats were atrophied, reduced in size, and weighed about half as much as the adrenal glands of the control rats.

DISCUSSION

The adrenocortical carcinoma under study induces atrophy of the adrenal glands of the tumor-bearing rat (5). This suggested secretion of physiologically active steroid hormones by the tumor. Polyuria and nitremia observed in these tumor-bearing rats pointed to a possible secretion of mineralocorticoids by this carcinoma.

The importance of the adrenal cortex in potassium metabolism was shown by chemical studies of the blood and tissues by Zwemer and Truskowski (6) and a quantitative relationship between a dose of cortical hormone and the percentage of survival of adrenalectomized mice following injection of KCl was established by Truskowski and Duszynska (7). On the basis of these studies, Kolmer (8) suggested a potassium tolerance test for clinical use to determine the degree of adrenocortical deficiency in doubtful cases. The rat was used as a test animal by Feil and Dorfman (9) to evaluate the potency of cortin like material excreted in urine.

Potassium tolerance test evaluates the effective concentration of corticosteroid (mineralocorticoids) in the test animal. In this study the carcinoma suspected of secreting corticosteroid was grown in the test animal. Thus effective concentration of corticosteroid in these animals was a resultant of the rate of secretion and the rate of its destruction or elimination. The response of Osborne-Mendel rats, intact and adrenalectomized, to KCl stress was similar to that obtained by Collins (10) in albino rats. Adrenalectomized rats with tumor tolerated 0.16m KCl, a dose always lethal to comparable rats without tumor, giving a qualitative proof of corticosteroid secretion by the tumor. Inability of the test rats to tolerate

the same dose of KCl after removal of the tumor added strength to this proof. Tumor-bearing rats did not tolerate any dose of KCl higher than 0.16m, irrespective of the size of the tumor. This means that corticoid concentration in tumor-bearing rat is maintained at the same level as in intact control rats, and suggests that other factors besides the secretory activity of the tumor play a part in regulating this level. The largest accessory adrenal bodies (30 mg.) did not protect the rat against 0.16m dose of KCl.

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THE INFLUENCE OF THYROTROPHIC HORMONE AND METHYL-THIOURACIL ON THE "CYSTEINYLTYROSINASE" (AND PROTEINASE) ACTIVITY OF THE THYROID GLAND OF THE RAT

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ABSTRACT

A method of assay of "cysteinyltyrosinase" activity applicable to the rat thyroid has been devised and the variations of this and of proteinase activity studied following the administration of thyrotrophic hormone or methylthiouracil. It was found that, in the case of "cysteinyltyrosinase," there was a significant increase in the total activity per thyroid gland, either as such or when related to unit body weight, but the *concentration* was significantly decreased. However, although the proteinase showed significant variations similar to those of "cysteinyltyrosinase" following administration of methylthiouracil, thyrotrophic hormone caused significant increases in total activity and in activity related to unit body weight and a trend towards *higher* values for concentration of proteinase.

IT IS generally considered that the release of the thyroid hormone from its stored form in thyroglobulin is mediated by proteolytic enzymes of the gland. Laver and Trikojus (1) have shown the complex nature of this proteolytic system and were able to effect a partial separation into a proteinase and peptidases. The proteinase component, apart from acting on haemoglobin, also split L-cysteinyl-L-tyrosine alone among a number of peptides tested. Later work has shown that the "cysteinyltyrosinase" (CTase) component can be separated from the proteinase and that other cysteine-containing peptides such as L-cysteinyl-L-phenylalanine and L-leucyl-L-cysteine are also readily hydrolysed in the presence of the enzyme (unpublished observations).

Recently, Watson and Trikojus (2)[1] reported that the proteolytic activity of the thyroid gland of the rat, as estimated with haemoglobin as substrate, is altered from the normal value when the gland is acted upon by thyrotrophic hormone (TSH) or an anti-thyroid drug. In the investigation described here, a method of assay of CTase activity, applicable to the rat thyroid, has been devised and the variations of this activity studied following the administration of TSH or methylthiouracil. Proteinase estimations (haemoglobin substrate) were also carried out.

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[1] In each of the three Tables in this paper the values in Column 7 should relate to 10 mg. *not* 100 mg. thyroid. The validity of the conclusions is, however, not affected.

MATERIALS AND METHODS

A crossed breed of male rats of the Wistar and *Rattus norvegicus* strains was used throughout. Stock animals were maintained in a room at 25° C and were fed a diet of Fido dog cubes[2] and water *ad lib.* with greens weekly. Groups of animals were selected for experiments such that the means and standard deviations of the weights of the groups were approximately the same.

L-Cysteinyl-L-tyrosine (CT) was synthesized by the method of Harington and Pitt Rivers (3) with minor modifications. The product was twice recrystallized from water, and gave a single spot following paper chromatography in *n*-butanol/acetic acid/water (4:1:1, v/v) in the presence of N-ethyl maleimide. Assay of the SH content by the method of Benesch and Benesch (4) gave the value of 91% SH. A Parke-Davis preparation of thyrotrophic hormone was used containing 9–10 Junkmann-Schoeller (J-S) (5) units/mg. Reagents were of A.R. grade.

Proteinase activity was estimated by the method of Anson (6) with minor modifications: 0.5 ml. of enzyme solution (diluted thyroid brei) was incubated with 2.5 ml. of 2% haemoglobin for 2 hours at 37° C at pH 3.5; the supernatant, following precipitation with trichloroacetic acid, was assayed with the phenol reagent of Folin-Dennis and results expressed as m.eq.$\times 10^4$ of tyrosine liberated.

CTase activity was assayed as follows: 60 μl. samples of thyroid brei, prepared from fresh tissue using 0.1M sodium acetate buffer pH 5.0, were incubated in stoppered tubes at 37° C with 60 μl. 40 mM peptide (CT). Enzyme-buffer controls were included. 21 μl. samples were withdrawn after 5, 25, 45 and 65 min. and delivered onto pieces of washed chromatography paper (7), 0.50$\times$1.25 cm., contained in reaction tubes calibrated at 10 ml. 2 ml. of the quantitative ninhydrin reagent of Connell, Dixon and Hanes (7) was then added to each tube and the tubes immersed in a boiling water bath for 20 min. After cooling, aqueous ethanol (1:1, v/v) was added to the 10 ml. mark and the light absorption read in a Beckman spectrophotometer (Model DU) at 570 mμ using a 1 cm. light path. Preliminary work had shown that, with the concentrations used, these assays were carried out at substrate saturation.

The pH-activity curve of the enzyme, studied over the range pH 3.5 to 6.5, reached a maximum at approximately pH 4.5 and then continued almost flat. Values were not sought on the alkaline side in view of the sensitivity of the substrate. The enzyme itself was found to be completely inactivated after a few minutes above pH 9.

EXPERIMENTAL

Effects of Methyl-Thiouracil and TSH on CTase and Proteinase Activity of Thyroid

The effects of methyl-thiouracil were studied with 26 rats divided into two groups. One group received methyl-thiouracil as a 0.05% aqueous solution in place of their drinking water for 14 days; the control group received water alone. All rats were accommodated singly and, at the end of the experimental period, were killed with ether, the thyroids dissected and weighed and breis of 0.8 ml. prepared with 0.1M sodium acetate buffer, pH 5.0. Four 60 μl. samples were removed for duplicate assays of CTase activity, the residue diluted with 0.9 ml. distilled water and two further samples of 0.5 ml. taken for single assays of proteinase activity.

In the second experiment 16 rats were divided into two groups. The treated group received subcutaneous injections of TSH twice daily (each injection 25 J-S units/100 gm. body weight in 0.5–1.0 ml. saline) for 5 days. The control group received injections of normal saline. On the sixth day the rats were killed with ether and thyroid breis prepared and assayed as described for the first experiment.

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[2] Barastoc Products, Melbourne.

TABLE 1. "CYSTEINYLTYROSINASE" AND PROTEINASE ACTIVITIES OF THE THYROID GLAND OF THE RAT. EFFECT OF METHYL-THIOURACIL—0.05% IN DRINKING WATER FOR 14 DAYS

Group	No. animals	Mean final body wt. (gm.)	Mean thyroid wt. (mg.)	"Cysteinyltyrosinase" Activity* per thyroid (a)	"Cysteinyltyrosinase" Activity per mg. thyroid (b)	"Cysteinyltyrosinase" Activity per gm. body wt. (c)	Proteinase log Activity** per thyroid	Proteinase log Activity per 100 mg. thyroid	Proteinase log Activity per 100 gm. body wt.
Me-thiouracil	13	180	49	.369	.0075	.00206	1.5930	1.9128	1.3399
Control	13	184	17	.301	.0180	.00167	1.4053	2.1730	1.1442
				Critical Differences (Proteinase)		5%—	.0685	.0557	.0628
						1%—	.0928	.0755	.0851

* Increase in log intensity per hour (see text).
** As m.eq. $\times 10^4$ tyrosine liberated.
The differences between the pairs of values under (a) (b) and (c) are all highly significant; F = 30.3, 149.4 and 20.3 for (a) (b) and (c) respectively with 1, 72 degrees of freedom in each case.

Differences between the means of the enzymic activities in the groups of animals in the two experiments were tested for significance by the analysis of variance. The CTase activities were calculated by graphing the logarithms of the light absorption intensities (Beckman values) against time (5, 25, 45, 65 min.). The activity was defined as the slope of the log intensity by time graph (i.e. increase in log intensity per hour).

RESULTS AND DISCUSSION

The results of the experiments are set out in Tables 1 and 2. In the case of CTase, the total activity per thyroid gland, either as such or when related to unit body weight, was found to be significantly increased following the administration of methylthiouracil or TSH. By contrast, the *concentration* of CTase in the gland was significantly decreased as a consequence of either treatment. However, although the proteinase showed significant variations similar to those of CTase following the administration of methyl-thiouracil, TSH caused significant increases in total activity and in activity related to unit body weight, and a trend towards *higher* values for concentration of proteinase. This finding is in accord with the results of Watson and Trikojus (2) who, using a larger dosage of TSH (2×50 units per day compared with 2×25 units in the present work), obtained a significant increase in proteinase concentration.

Thus, at least two of the components of the proteolytic system of the thyroid gland respond to changes induced in the physiological status of

TABLE 2. "CYSTEINYLTYROSINASE" AND PROTEINASE ACTIVITIES OF THE THYROID GLAND OF THE RAT. EFFECT OF THYROTROPHIC HORMONE—25 UNITS/100 GM. BODY WEIGHT TWICE DAILY (SUB-CUT). FOR 5 DAYS

Group	No. animals	Mean final body wt. (gm.)	Mean thyroid wt. (mg.)	"Cysteinyltyrosinase" Activity* per thyroid (a)	"Cysteinyltyrosinase" Activity per mg. thyroid (b)	"Cysteinyltyrosinase" Activity per gm. body wt. (c)	Proteinase log Activity** per thyroid	Proteinase log Activity per 100 mg. thyroid	Proteinase log Activity per 100 gm. body wt.
TSH	8	146	25	.340	.0135	.00234	1.5609	2.1616	1.3995
Control	8	155	17	.299	.0181	.00193	1.3368	2.1171	1.1476
				Critical Differences (Proteinase)		5%—	.0812	.0766	.0612
						1%—	.1127	.1063	.0849

* Increase in log intensity per hour (see text).
** As m.eq. $\times 10^4$ tyrosine liberated.
The differences between the pairs of values under (a) (b) and (c) are all highly significant; F = 11.6, 42.3 and 40.1 for (a) (b) and (c) respectively with 1, 42 degrees of freedom in each case.

the gland although there are differences in the direction of this response. Further discussion of the relevance of these observations to thyroid physiology must await clarification of the specific roles of these enzymes in the thyroid gland. Current investigations in this Department are concerned with the preparation of proteinase, CTase and carboxypeptidase (1) free from mutual contamination, and with a study of their actions, both independently and in combination, on appropriate thyroid substrates.

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NOTES AND COMMENTS

FAILURE TO DEMONSTRATE TSH-RELEASING ACTIVITY OF PITRESSIN AND OXYTOCIN[1]

ABSTRACT

In these experiments, Pitressin and oxytocin were investigated as substances that might possibly act to release TSH from the pituitary gland in a manner analogous to the corticotrophin releasing factor. Thyrotrophin release was inferred from *in vivo* measurements of thyroid I^{131} release rate in three types of rat preparations: 1) normal animals, 2) adrenalectomized rats maintained on 0.9% sodium chloride in the drinking water and cortisone 2.5 mg./day, 3) rats with lesions in the "thyrotrophic" area of the hypothalamus. Drugs were given subcutaneously in 25% polyvinylpyrrolidone solution. It was found that Pitressin in dosage up to 500 mU. every 8 hours and oxytocin in dosage of 233 mU. every 8 hours failed to influence thyroid activity

Hypothalamic influences on the regulation of thyrotrophic activity have been shown by numerous studies (1), but the mechanism through which this control operates has not been elucidated. One explanation of this phenomenon is that a chemical of hypothalamic origin is secreted into the hypophysial-portal circulation whence it reaches and stimulates the adenohypophysis (2). Although there is reasonably good evidence that this general kind of system is involved in the regulation of adrenocorticotrophin (3), the existence of an analogous neurohumoral factor controlling TSH has not been established. In a previous experiment Pitressin (or a non-vasopressin constituent of Pitressin) was investigated as a possible regulator of TSH release (4), but no evidence of this action was found. The object of the present study was to re-examine the effects of Pitressin on thyrotrophin release and to determine if the related hormone, oxytocin, possessed TSH releasing activity.

METHODS AND RESULTS

Adult male rats of the Holtzman strain were used. They were given Purina laboratory chow and water *ad libitum*, and were housed in a controlled temperature room at $26 \pm 1°$ C. Thyroid function as determined by *in vivo* measurement of I^{131} release rate (4) was taken as an indication of TSH release. Adrenalectomized animals were operated upon through a conventional dorsal approach and maintained on 2.5 mg. cortisone per day I.P. and physiological saline *ad libitum* from the time of operation. Rats with hypothalamic lesions were prepared as previously described (1). They manifested low baseline thyroid activity as well as other hypothalamic dysfunctions such as hyperphagia and diabetes insipidus.

Preliminary experiments were done to establish the time course of antidiuretic effect of Pitressin[2] as a guide to choice of dosage in the study of effects on thyroid function. Pitressin was dissolved in saline, 25% polyvinylpyrrolidone[3,4] (PVP), or a gelatin solu-

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[2] Furnished through the kindness of Dr. D. A. McGinty, Parke, Davis and Company, Detroit, Michigan.

[3] The use of this substance as a vehicle for polypeptide hormones was suggested to us by Dr. Roger Guillemin, Houston, Texas.

[4] Furnished through the kindness of Dr. Arnold E. Osterberg, Abbott Laboratories, North Chicago, Ill.

tion (5), or was administered as Pitressin Tannate in oil.[2] Antidiuretic activity was measured by the technique of Wilson and McGinty (6). A subcutaneous injection of 15 mU. of Pitressin in PVP delayed the usual diuretic response to water loading by 6 to 7 hours; a similar dose in saline delayed diuresis by 72 minutes. When administered in gelatin, the time course of Pitressin effect was roughly the same as with PVP and as the Tannate compound in oil given intramuscularly, the effect was prolonged, and more complete for at least 8 hours. Because repeated intra-muscular injections of peanut oil were found in a previous study to produce moderate inhibition of thyroid I^{131} release, presumably through a stress-producing action (4), PVP was chosen as the vehicle in this experiment, although gelatin solution would probably have served as well. In experiments on thyroid function, Pitressin or synthetic oxytocin[5] were administered subcutaneously in 0.5 ml. of PVP solution.

1. *Thyroid Response of Normal Rats*

I^{131} release curves were determined in 40 rats weighing between 210 and 270 gm. Beginning immediately after the initial neck count they were injected subcutaneously every eight hours with one of the following substances:

Group 1, (10 rats) 300 mU Pitressin in 0.5 ml. 25% PVP
Group 2, (10 rats) 233 mU oxytocin in 0.5 ml. 25% PVP
Group 3, (10 rats) 0.5 ml. 25% PVP
Group 4, (10 rats) 0.5 ml. saline

Injections were continued for 80 hours. The slopes of release curves plotted from the mean neck counts were as follows: Saline control 20.0%/day. PVP control 15.5%/day, Pitressin 16.5%/day, oxytocin 17.7%/day. The differences between the curves were small and no evidence of acceleration of release was obtained with either oxytocin or Pitressin. The small degree of slowing caused by PVP injections may be evidence of a stressful effect.

2. *Thyroid Response of Adrenalectomized Rats*

Adrenalectomized rats were used because of the observation of Harris and Woods (7) that activation of ACTH by electrical stimulation of the rabbit hypothalamus interfered with TSH release; it was considered possible that corticoid releasing effects of Pitressin (3) might interfere with TSH releasing effects.

I^{131} release curves were determined in 29 adrenalectomized rats (380–480 gm.) over a 70 hour period. Pitressin in PVP, 500 mU. every 8 hours, was administered to 14, 8 received PVP and 7 saline. Mean release rate of the Pitressin-treated group was 18.8% per day, of the PVP treated 20.0% per day and of the saline treated 22.0% per day. Again, differences were small, no evidence of acceleration was observed and there was a suggestion that PVP alone caused a slight slowing of I^{131} release rate.

3. *Thyroid Response of Rats with Hypothalamic Lesions*

Rats with lesions were used because their low baseline thyroid activity would have made activation by a TSH releasing substance more readily apparent. Furthermore, destruction of the neural areas which influence TSH might have been expected to sensitize the preparation to neurohumoral effects.

Fifteen rats with lesions were observed. Each animal served as its own control for five days, receiving PVP only every eight hours. On the fifth day the administration of Pitressin in PVP 300 mU. (8 rats) and oxytocin 233 mU. (7 rats) was begun and continued for 72 hours. Release rates determined from the mean neck counts during the

[5] Supplied as "Syntocinon" through the kindness of Sandoz Laboratories.

first five days were 8.0 and 6.8% per day in the Pitressin and oxytocin group respectively. Three additional counts were obtained after the start of treatment and these fell on the projection of the curve drawn for the first five days, thus indicating no significant effect of the treatment.

DISCUSSION

No evidence of a TSH-releasing effect of oxytocin or of Pitressin in the dosage tested was obtained in this experiment. With respect to Pitressin this finding confirms the results of a previous study (4). In experiments in which plasma protein bound iodine was used as a measure of thyroid function in rats, a similar failure to produce activation after the administration of vasopressin (80 mU/100 gm./day) was recorded by Arimura *et al.* (8). In contrast to these negative results several investigators have reported evidence of thyroid stimulation following Pitressin or oxytocin injections. Fraja and Martini (9), who assayed thyrotrophin by a chick thyroid microhistometric technique, found that either Pitressin (0.2 U/kg.) or oxytocin (0.2 U/kg.), when injected into the subarachnoid space of dogs, induced an elevation of serum TSH levels. In this system oxytocin was the more effective drug. In rabbits Pitressin injected intravenously (0.2 U/kg.) is reported to have caused a rise in serum TSH as assayed by an *in vitro* thyroid slice I^{131} uptake method (10). The effects of prolonged treatment with Pitressin on thyroid I^{131} uptake are not well established. In rats uptake is said to increase (11) while in mice no stimulation is observed (12). Note should be made of the report that Pitocin has slight thyroid stimulating action in guinea pigs (13), one ml. containing the activity equivalent of about 5 μg. U.S.P. Thyrotrophic Reference Substance. Contamination of the Pitocin with TSH cannot be excluded, however.

Although Pitressin, vasopressin and oxytocin have been studied most extensively as possible TSH releasing substances, consideration has been given to other substances of hypothalamic origin, again with conflicting results. Water extract of the anterior hypothalamus of the dog when injected into rats is said to produce thyroid enlargement, elevation of the plasma protein bound iodine level, and increase in plasma thyroid stimulating activity (14). In further observations by the same group, similar hypothalamic extracts caused thyroid enlargement, increased radioiodine uptake and increased thyroid/serum iodide ratio, both in normal rats and in those with hypothalamic lesions (15). By contrast, hypothalamic tissue has failed to stimulate the production of TSH by pituitary tissue grown *in vitro* (16), although such preparations release ACTH. It must be concluded that the identity, or even the existence of a hypothalamic neurohumour with TSH releasing properties remains an unsettled problem.

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FURTHER STUDIES ON THE PLASMA PROTEIN-THYROID HORMONE COMPLEX[1]

ABSTRACT

The plasma protein-thyroid hormone complex was investigated by (1) the binding (paper and starch gel electrophoresis) of I^{131}-L-thyroxine and I^{131}-L-triiodothyronine by plasma proteins, (2) the passage of T4 and T3 from such protein linkages across a dialysis membrane and (3) the *in vitro* incorporation of T4 and T3 by human erythrocytes. Results indicate (1) electrophoretic heterogeneity of the 'albumin' and 'inter-alpha globulin' serum proteins which preferentially bind thyroxine, (2) a major effect of Tris (hydroxymethylaminomethane) buffer on both the overall thyroxine binding capacity of serum and the relative T4 binding by the 'pre-albumin' and 'inter-alpha globulin,' (3) a significant effect of pH on binding, (4) a striking inhibitory effect of Veronal buffer on T4 binding by serum albumin.

Recent studies attest to the growing interest in, and complexity of, the binding of thyroid hormones by plasma proteins. In addition to the specific thyroxine (T4)-binding inter-alpha globulin (TBG) and the secondary carrier, albumin, the presence of a specific pre-albumin T4-binding protein (TBPA) has been indicated in various electrophoretic systems (1–5). Additional studies have (a) ascribed a primary physiologic role to 'free' thyroxine (6), (b) reported the presence of an iodine compound associated with albumin in the plasma of certain thyrotoxic but not euthyroid subjects (7) and (c) suggested that T4 binding properties of TBG are due to TBPA present as a complex of unknown composition (8). This report deals with certain results of concomitant studies of (a) the plasma protein binding (paper and starch gel electrophoresis) of I^{131}-L-T4 and I^{131}-L-triiodothyronine (T3), (b) the passage of T4 and T3 from such protein linkages across a dialysis membrane and (3) the *in vitro* incorporation of T4 and T3 by tissues.

METHODS

Electrophoresis. I^{131}-L-T4 and I^{131}-L-T3, in varying amounts of stable carrier (1–200 μg.%), were added to either plasma, serum, various serum fractions (Cohn), or highly purified inter-alpha globulin (9), permitted to stand for 20 minutes to 18 hours at 6° C, subjected to paper or starch gel electrophoresis by the method of Smithies (10). Different buffers (Veronal, borate, phosphate) of varying molarities and pH's were employed. Distribution of radio-

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activity was determined by (1) direct strip or gel counting, (2) cutting strip or gel into 1 cm. segments and counting segments in a well-type scintillation counter, and (3) radioautography (with paper strips only). For protein distribution, paper strips were stained by bromphenol blue, gels by the method of Smithies. The areas under the various peaks were determined planimetrically. Combinations of paper and starch gel electrophoresis were employed. For example, specific segments (1 cm.) of a paper strip after electrophoresis were inserted into a slit in a starch gel and re-electrophoresed using the same or different buffers. Alternatively, the contents of 1 cm. segments of the starch gel after electrophoresis were removed by freezing, thawing and squeezing, followed by re-electrophoresis of the eluate on paper or on another starch gel using e.g. different buffer.

In vitro binding capacity. Human red blood cells, washed five times with isotonic saline, were resuspended, in amounts yielding a hematocrit of 40–50%, in saline, various buffers, plasma, serum, plasma fractions (Cohn), highly purified inter-alpha globulin solutions or starch gel segment eluates. Tracer amounts of T4 or T3 were added, incubated with shaking at 37° C for 1–6 hours, the rbc then washed to remove loosely adherent radioactivity, and the incorporated radioactivity determined in a well-type scintillation counter (within ±2% accuracy). Results are expressed as % uptake, viz:

$$\frac{\text{counts per minute in rbc} \times 100}{\text{total counts in medium per minute}}$$

and corrected to 100 hematocrit.

Dialysis. Tracer amounts of T4 or T3 were added to different concentrations of various protein containing solutions. One to three ml. of such solutions was placed in dialysis tubing (Visking cellulose-8/32″) which was then tied at both ends. The bag, with or without prior dialysis against saline or buffer, was then placed in an Ehrlenmeyer flask containing 10 ml. of varying solutions (different buffers or any other of above protein solutions) and agitated at 37° C up to 28 hours. At varying intervals, 1 ml. aliquots of the surrounding medium were removed, radioactivity determined, and returned to flask. Determinations were thus made of both the absolute amount of T4 or T3 transferred at specific time intervals and the over-all rate of transfer (slope of curve). Control studies revealed that, although T4 or T3 adhered to dialysis tubing when saline or buffer media were used, 100% of initial radioactivity was accounted for at varying time intervals in medium plus bag contents when protein was present.

RESULTS

On paper or starch gel electrophoresis of serum in Veronal buffer (pH 8.6, 0.1M), I^{131} T4 was found principally in the inter-alpha globulin area, with a small amount in the proximal region of the leading albumin band. When the paper segment containing the albumin-localized T4 was re-electrophoresed on starch gel (borate buffer, pH 8.6, 0.3M) the radioactivity was found distinctly separable from the broad, heavily staining albumin band, being sharply localized in a narrow band closer to the origin in which no stainable protein could be found. Similarly, re-electrophoresis on starch gel of the inter-alpha globulin band from paper resulted in resolution of radioactivity and associated protein into two and sometimes three distinct bands.

On starch gel electrophoresis of serum in Tris (hydroxymethylaminomethane) buffer (pH 8.6, 0.5M) I^{131} T4 (tracer amounts) was found in three sites—(1) the inter-alpha globulin area, (2) a pre-albumin band, and (3) a small amount in the albumin band. No pre-albumin binding of I^{131} T3 was demonstrable. The ratio of T4 bound

$$\frac{\text{(TBG-T4)}}{\text{TBPA-T4}}$$

was compared in nine euthyroid and ten hyperthyroid patients. Whereas repeated determinations in a given subject were in close agreement, there was a high degree of variability in different subjects resulting in complete overlapping of values between the euthyroid (ratios of 0.55 to 1.8) and hyperthyroid (0.21 to 2.4) series. The effects of varying the experimental conditions were then studied. In addition to the difference between Veronal and Tris (no consistent pre-albumin binding of T4 with Veronal), the molarity of the Tris buffer was found to be a major determinant of the pattern of binding distribution. The ratio of

$$\frac{\text{TBG-T4}}{\text{TBPA-T4}}$$

could be varied at will, even reversed, by altering the molarity of Tris employed.

The significant role of Tris was further indicated by a study of the pre-albumin band eluted from the starch gel. The T4 binding capacity of this eluate was estimated by its ability (1) to decrease the uptake of I^{131} T4 by washed rbc as compared to saline or plasma and (2) to alter the transfer of I^{131} T4 across a dialysis membrane against albumin or plasma. The binding capacity was lost following its dialysis against H_2O, saline, or Krebs-Henseleit buffer. The binding capacity of the bag contents could then be completely restored by re-dialysis against Tris buffer and the molarity of the Tris used for re-dialysis determined the resultant binding (Table 1). Extensive dialysis of TBG fraction did not affect its binding capacity.

Finally, the direct addition of Tris to plasma increased its total T4 binding above its native capacity as measured by the rbc uptake in whole blood; the degree of increased binding varied with the molarity of Tris added (Table 2). Similarly, the addition of Tris to whole plasma increased its binding capacity for T4 above native plasma in the dialysis system (Table 3). Tris had no effect on T3 binding or dialysis transfer.

TABLE 1. EFFECT OF PRE-ALBUMIN* ON I^{131} T4 UPTAKE BY WASHED RED BLOOD CELLS

Test system	% uptake (per 100 hematocrit)
I. Control (rbc in Saline)	134
II. 0.2 ml. Pre-albumin	
A. Eluate before dialysis	55
B. After dialysis against saline	136
III. 0.2 ml. Pre-albumin (II-B) after redialysis against:	
0.05M Tris Buffer	116
0.10M Tris Buffer	109
0.30M Tris Buffer	79
0.50M Tris Buffer	53
0.60M Tris Buffer	43

* Eluted from starch gel after electrophoresis of euthyroid serum (0.5M Tris Buffer, pH 8.6).

TABLE 2. EFFECT OF TRIS BUFFER ON *in vitro* rbc UPTAKE OF I^{131} T4 FROM WHOLE BLOOD (2 HOURS)

Molarity of added tris	% uptake/100 hematocrit
0 (control blood)	2.19
0.06	1.78
0.125	1.53
0.25	1.10
0.50	1.09

TABLE 3. EFFECT OF TRIS BUFFER IN PLASMA ON TRANSFER OF I^{131} T4 FROM HUMAN SERUM ALBUMIN ACROSS DIALYSIS MEMBRANE*

Composition of medium surrounding dialysis bag	% I^{131} T4 transferred to plasma	
	3 hrs.	22 hrs.
1 ml. Plasma + 9 ml. 0.85% Saline	15.8	42.2
1 ml. Plasma + 9 ml. .05M Tris Buffer	16.2	45.1
1 ml. Plasma + 9 ml. 0.2M Tris Buffer	21.7	61.3
1 ml. Plasma + 9 ml. 0.5M Tris Buffer	23.5	71.0

* Bag Contents = 2 mgm. human serum albumin; .012 ugm I^{131} T4, 1.0 ml. 0.85 saline.

TABLE 4. EFFECT OF VARIOUS BUFFERS ON TRANSFER OF I^{131} T4 FROM HUMAN SERUM ALBUMIN ACROSS DIALYSIS MEMBRANE*

Composition of medium surrounding dialysis bag	% I^{131} T4 transferred	
	4 hrs.	22 hrs.
10 ml. Krebs-Henseleit Buffer (pH = 7.4)	11.7	20.4
10 ml. 0.2M Tris Buffer (pH = 8.6)	11.8	20.0
10 ml. 0.2M Phosphate Buffer (pH = 8.7)	10.5	19.3
10 ml 0.2M Veronal Buffer (pH = 8.6)	38.2	60.6

* Bag Contents = 2 mg. human serum albumin; .012 ug. I^{131} T4, 1 ml. 0.85% saline.

Alterations in pH similarly affected T4 binding and dialysis transfer. Whereas binding and transfer were essentially unchanged over a wide range about physiological values (pH 7.1 to 7.8), there was a decrease (reversible) in binding at pH levels below 7.0 and a striking increase (partially irreversible) of binding at pH's of 8.2–9.6, including the 8.6 used so frequently in electrophoresis.

It was of some interest that although Veronal buffer (pH 8.6, 0.1M) had no effect on total plasma T4 binding in the red cell system, it caused a striking inhibition of T4 binding by albumin in the dialysis system (Table 4).

Such results indicate the need for careful evaluation of the effects of technical factors in attempting physiological assessments of the protein binding of thyroid hormones.

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VASCULAR EXCLUSION OF THE LIVER: ITS INFLUENCE ON GLUCOSE METABOLISM[1]

ABSTRACT

When rats are prepared with vascular by-pass of the liver the level of glucose in the blood progressively decreases. This hypoglycemia is not prevented by the administration of epinephrine. When insulin is administered to the by-passed animals

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there is a continuous decrease in the level of blood glucose without restoration towards pre-injection levels as occurs in the control animals. Moreover, half of the animals die from hypoglycemia in the by-passed group whereas no deaths occur in the control. These findings indicate that vascular by-pass successfully prevents the liver from exerting its actions on the above aspects of glucose metabolism.

In a recent publication the author reported a relatively simple technique for excluding the liver from the active vascular circulation of an intact rat (1). This preliminary investigation showed that the by-pass was effective since the animal failed to produce bile or clear injected bromsulphalein from the peripheral circulation. The present study was undertaken to submit this preparation to further exploration and will demonstrate that the vascular by-pass also effectively prevents the liver from exerting its known actions on certain aspects of glucose metabolism.

METHODS

Male rats of the Long-Evans strain which weighed approximately 350 grams were employed. Animals with hepatic vascular by-pass were prepared by hepatic arterial interruption, bile duct cannulation and portal-caval shunt, as described in a previous communication (1). Control animals were subjected to bile duct cannulation only. Food was withheld from all animals for a period of 18 hours. The anaesthetic employed was nembutal, administered by intraperitoneal injection. The determination of plasma glucose was made initially and at thirty minute intervals on 0.2 cc. of venous blood, according to the method of Saifer and Gerstenfeld (2).

Three experiments were performed using equal numbers of paired rats, each pair consisting of a "hepatic by-passed" and control animal. In the first experiment the changes in the level of glucose were compared in paired animals for a period of 90 minutes after the operation. The second experiment was similar, except that 0.15 cc. of 1/1000 epinephrine was injected subcutaneously immediately after removal of the initial blood sample. In the third experiment, instead of epinephrine, 0.125 unit of insulin per 100 gm. of body weight was injected intravenously immediately after removal of the initial blood sample. The data are expressed as the mean changes in the plasma level of glucose with time.

RESULTS

Experiment 1 (Fig. 1) demonstrated that in the "hepatic vascular by-passed" rats without epinephrine, there was a continuous decline in blood glucose level during the 90 minutes of the experiment, while the control group showed a steady rise. From these curves it is suggested that in the by-passed group the liver was unable to release glucose into the peripheral circulation and a progressive fall in the blood glucose level resulted. The rise in blood glucose observed in the control group has been described with nembutal anaesthesia (3).

In Experiment 2 following the injection of epinephrine (Fig. 1), the by-passed rats showed a fall in the level of blood glucose which paralleled that of the by-passed group in Experiment 1, and at the end of 90 minutes there was no statistical difference in the changes in glucose level in the by-passed animals with or without epinephrine ($P < 0.1$). The control group receiving epinephrine demonstrated the expected marked rise in plasma glucose which is presumably due to the conversion of hepatic glycogen to glucose. This experiment further suggests that the by-passed liver is unable to release glucose into the peripheral circulation even after the administration of epinephrine. The finding is similar to that reported in hepatectomized dogs where in spite of epinephrine a progressive hypoglycemia occurred (4).

In Experiment 3 following the injection of insulin (Fig. 2), in the by-passed group the fall in blood glucose was 70 mg.% in one half hour and the level continued to decrease to 98 mg.% at the end of the 2 hour period. Moreover, at this time 3 animals died from hypoglycemia. This was not observed in the control group in which the blood sugar fell 53 mg.% in one half hour and at the end of two hours there was an expected slight recovery (9 mg.%) towards pre-injection levels. The usual tendency of the blood glucose to return towards the pre-injection level after the administration of insulin was

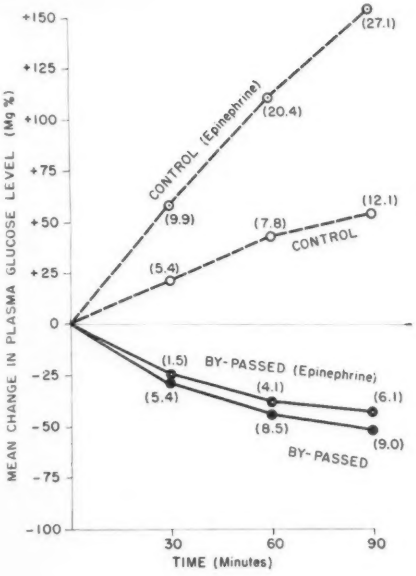

FIG. 1. Mean changes in plasma glucose with time, in vascular by-passed animals and controls, with and without epinephrine. *Note:* each point on the curve is the arithmetic average of determinations in 6 animals and the standard deviation is in parenthesis.

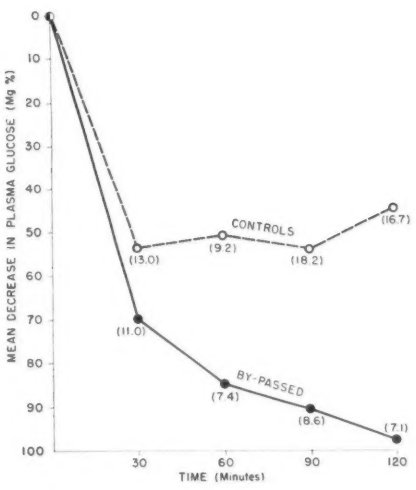

FIG. 2. Mean decrease in plasma glucose with time, in vascular by-passed and control animals following the administration of insulin. *Note:* each point on the curve is the arithmetic average of determinations in 8 animals and the standard deviation is in parenthesis.

abolished in the "by-passed" animals. Again this finding is the same as was reported in the hepatectomized animal (5).

Since it has now been demonstrated that vascular by-pass effectively prevents the liver from exerting its known actions on glucose metabolism as well as preventing bile production and bromsulphalein clearance, it would appear that this intact preparation, which is far less traumatized than the hepatectomized-eviscerated rat, should be of great value for studying the role of the liver in metabolic processes.

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PROTEIN ANABOLIC ACTION OF TESTOSTERONE PROPIONATE IN HYPERTHYROID-CASTRATED RATS[1,2]

ABSTRACT

dl-Thyroxine, 10 μg./day, produced in adult castrated rats on constant food intake an initial increase in protein catabolism which disappeared after six days. The body weight, however, continuously decreased during the 21 days of treatment. The simultaneous administration of testosterone propionate, 2.5 mg./day, abolished the initial protein catabolism and produced an effect on urinary nitrogen excretion identical with that in non-thyroxine treated rats. The body weight change produced by the androgen was not altered by the simultaneous administration of thyroxine.

Thyroxine in excess is known to produce loss in body weight and an increase in protein catabolism when the energy demands are not met (1, 2). Androgen on the other hand produces an increase in body weight due to more efficient utilization of dietary protein (3, 4). It seemed of interest to determine whether the androgen could produce its protein anabolic action in the presence of the extra caloric demands induced by an excess of thyroxine. The protein anabolic action of testosterone propionate has been shown in thyrotoxic patients who had been stabilized by increasing the calories, protein and vitamins in their diet (5).

METHODS

Rats (Sprague-Dawley) were maintained in individual metal metabolism cages in an air-conditioned room at 25.5–26.6° C and with artificial light regulated at 12 hours per day. Some of the animals were castrated at 5 weeks of age and the rest at 5 months of age. They were fed Rockland rat diet until about 7 months of age when they were transferred to the prepared diet used for metabolic studies (4). The food intake was regulated until the rats exhibited constant body weight and urinary nitrogen excretion. The rats were approximately 14 months old at the beginning of injections.

Crystalline DL-thyroxine, 2.5 mg., was dissolved in 1 ml. 0.2N sodium hydroxide and diluted to 25 ml. with water. A fresh solution was prepared weekly. The thyroxine, 10 μg./day, and testosterone propionate[3], 2.5 mg./day, were injected at 2:30–3:00 P.M. each day.

The routine for feeding, weighing and injections of the rats and methods of analyses were as described (3).

RESULTS

Body Weight: The testosterone propionate produced the expected (4) change in body weight and thyroxine produced a continuous decrease until injections were stopped (Fig. 1). The effect of the androgen on body weight was evident in spite of the decrease in body weight produced by the simultaneous injection of thyroxine (Fig. 1).

Nitrogen Excretion: Testosterone propionate produced the expected (4) effect on urinary nitrogen excretion (Fig. 1, Table 1). Thyroxine stimulated an extra excretion of nitrogen in the urine only during the first 6 days of injections. This phenomenon was abolished by the simultaneous administration of androgen. Indeed the urinary nitrogen excretion of the rats receiving both hormones was identical with that of the rat receiving only testosterone propionate. On cessation of injections the rats receiving only testosterone propionate lost a little more nitrogen than those receiving both hormones.

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[2] The experimental work was carried out at the Oklahoma Medical Research Foundation with the technical assistance of Sarah McConnell.

[3] The testosterone propionate was provided as Perandren, 50 mg./ml. in sesame oil and 0.5% chlorbutanol, by Ciba Pharmaceutical Products, Inc.

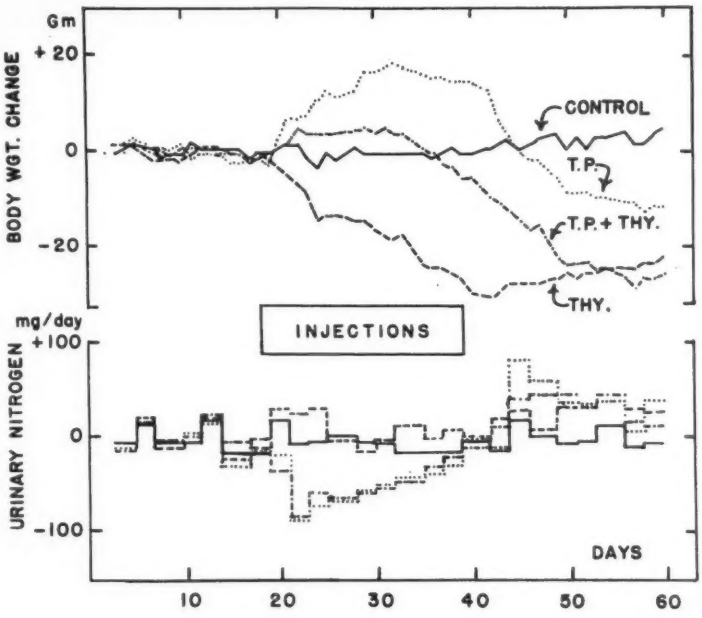

FIG. 1. Changes in body weight and urinary nitrogen excretion produced in castrated rats by the administration of testosterone propionate (T.P.), 2.5 mg./day, and DL-thyroxine (THY), 10 μg./day, singly and simultaneously. The changes in body weight and urinary nitrogen for the individual periods are from the averages of the preinjection periods which are indicated as zero (0).

DISCUSSION

The transient increase in protein catabolism after thyroxine administration indicates that the continuous loss in body weight was due to an increased rate of utilization of body fat to meet the extra caloric demands induced by the thyroxine. A prolonged negative nitrogen balance has been produced in a male and a female myxedematous

TABLE 1. EFFECT OF TESTOSTERONE PROPIONATE AND DL-THYROXINE ON THE NITROGEN METABOLISM AND BODY WEIGHT OF CASTRATED RATS. (5 RATS PER GROUP)

Treatment	Food nitrogen	Urine nitrogen[1]				Body weight		
		Pre-injn.[3]	Change[2]		Post-injn. total	Pre-injn.	Maximum change	Post-injn.
			Max.	Total			Injn. period	
	mg./day	mg./day	mg./day[4]	mg./20 days	mg./21 days	gm.	gm.	gm.
Sesame Oil, 0.1 ml./day	340	265	+ 3	− 97	−102	406	0	+ 3
DL-Thyroxine, 10 μg./day	334	252	+29	+ 280	+643	413	−29	+ 6
Testosterone Propionate, 2.5 mg./day	329	264	−64	−1023	+748	418	+18	−25
T.P. + DL-Thyroxine	326	253	−60	− 958	+669	393	+ 5	−21

[1] Fecal nitrogen 33 (25–40) mg./day.
[2] Change from preinjection values.
[3] Average of 16 days preceding injections.
[4] Average of 7 days (4th to 11th days) except the thyroxine experiment which is for 6 days (2nd to 8th days) (see Fig. 1).

patient but not in a normal male by a daily oral dose of 100 μg. triiodothyronine (6). Testosterone propionate caused nitrogen retention during the period of negative nitrogen balance produced in the male myxedematous patient by the triiodothyronine (6) and in a young man with severe thyrotoxicosis complicated by diabetes mellitus (7).

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ORGAN WEIGHTS OF ANIMALS MAINTAINED ON ALLEGED APHRODISIACS: "PEGA PALO" AND "BOIS COCHON"[1]

ABSTRACT

There is no evidence that either bois cochon bark or pega palo vine (alleged aphrodisiacs) exerts an effect on the testes, prostate, or seminal vesicles of hypophysectomized or intact rats. The absence of an increase in the weight of the testes in normal or hypophysectomized animals argues against a gonadotropic effect, and the failure to depress testicular size in intact rats or to restore the weights of the accessory genital structures after hypophysectomy excludes any possible androgenic action.

Alcohol in which the vine of *Rhyncosia pyramidalis* ("pega palo") or the bark of *Bursera gummifera* ("bois cochon") has been soaked is reputed to restore or increase sexual performance in the human male (1, 2). Objective evidence on this point is however lacking. The possibility that the vine or the bark possesses either gonadotropic or androgenic effects has therefore been explored in hypophysectomized and in intact male rats.

MATERIALS AND METHODS

Intact or hypophysectomized Sprague-Dawley rats, 100–150 gm. males, were fed ground rat chow (Wayne Laboratories) containing approximately 5 or 2.5 per cent ground pega palo vine, bois cochon bark, or kiln-dried sawdust. The various regimens employed are listed in Table 1. The animals were sacrificed by decapitation and exsanguination at the end of approximately two months. The organs listed in Table 1 were dissected, freed from connective tissue, blotted dry, weighed on a Roller balance with a sensitivity of 0.5 mg. and related to the final body weight.

RESULTS

Admixture of bois cochon bark or of pega palo vine to comprisè 2.5 to 5 per cent

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TABLE 1. MEAN ORGAN WEIGHTS AS PERCENTAGE OF BODY WEIGHT IN HYPOPHYSECTOMIZED AND INTACT RATS WITH OR WITHOUT BOIS COCHON OR PEGA PALO IN THE DIET

Expt. (Duration)	Regimen*	# of animals		Initial body wt. (gm.)	Mean daily food intake (gm.)	Mean daily H_2O intake (ml.)	Final body wt. (gm.)	Mean organ weights as % of final body weight									Semi-nal vesicles	Pros-tate
		Start	Finish					Thy-roid	Pituitary	Heart	Liver	Pancreas	Adrenals	Right kidney	Left kidney	Testes		
I. 79 days	Hypophysectomy +2.5% bois cochon†	13	10	119.6	6.35	45.8	144.6	.006		.335	3.61	.383	.006	.323	.298	.164	.009	**
79 days	Hypophysectomy +2.5% sawdust	12	8	109.5	7.52	40.5	141.3	.009		.349	3.78	.356	.010	.320	.311	.213	.012	**
II. 41 days	Hypophysectomy +2.5% bois cochon	12	11	119.2	14.1	24.1	126.6	.007		.354	3.57	.422	.007	.367	.360	.243	.011	.007
48 days	Hypophysectomy +house diet	12	12	111.5	13.7	20.9	129.9	.006		.422	3.33	.459	.008	.404	.399	.221	.011	.005
III. 37 days	Intact rats +2.5% bois cochon	12	12	147.4	13.8	19.9	212.3	.007	.004	.314	3.27	.408	.018	.387	.383	1.387	.167	.084
37 days	Intact rats on house diet	12	12	161.1	13.4	19.9	212.9	.007	.004	.348	3.02	.379	.016	.389	.389	1.61	.170	.082
IV. 56 days	Hypophysectomy +5% pega palo	12	8	111.2	8.7	38.9	134.9	.006		.365	4.20	.510	.007	.353	.340	.161	.014	.007
57 days	Hypophysectomy +5% sawdust	12	9	108.2	9.3	33.6	120.9	.006		.348	4.56	.457	.006	.362	.350	.164	.012	.006
V. 26 days	Intact rats on 5% pega palo	12	12	139.7	16.5	36.7	234.3	.009	.005	.359	3.58	.406	.017	.455	.455	1.250	.1809	.088
27 days	Intact rats +5% sawdust	12	12	143.3	16.7	32.6	204.1	.008	.005	.383	3.52	.374	.020	.467	.467	1.442	.1632	.086

* All hypophysectomized animals received a 5% solution of sucrose as drinking water and 7 grains of thyroid plus 1 mg. of cortisone were added to each kilogram of food. Non-hypophysectomized rats received distilled water.
** Too small to weigh.
† Attempts at 5% level of bois cochon in the diet produced anorexia and weight loss.

of the diet did not alter the weight of the testes, prostate or seminal vesicles of intact or hypophysectomized male rats (Table 1). Hypophysectomy produced the expected decrease in the size of these organs and of the adrenals but no discernible change was recorded in the weight of the thyroids. It is to be noted however that these were among the smallest of the organs weighed.

The ingestion of food, and of water and the weights of the heart, liver, pancreas and kidneys were not significantly altered by inclusion of the bark or vine in the diet.

DISCUSSION

There is no evidence that either bois cochon bark or pega palo vine exerts an effect on the testes, prostate, or seminal vesicles of hypophysectomized or intact rats. The inability of these agents to restore the weights of the testes, seminal vesicles and prostate of hypophysectomized animals argues against a gonadotropic effect, and the failure to depress testicular size in intact rats excludes any possible androgenic action.

As judged by the weights of the liver and kidneys in accordance with the criteria of Smyth *et al.* (3) these materials do not appear to be toxic when ingested at this level for periods up to two months in length. Neither was the relative size of the pituitary or pancreas altered.

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THE ENDOCRINE SOCIETY

1960 Annual Meeting

The Forty-Second Annual Meeting of The Endocrine Society will be held in the Eden Roc Hotel, Miami Beach, Florida, Thursday, Friday, and Saturday, June 9, 10, 11, 1960.

The Chairman of Local Arrangements is Dr. Kenneth Savard.

Scientific Sessions will be held from 9:00 A.M. to 5:00 P.M. daily and in addition there will be simultaneous afternoon sessions. The annual dinner is scheduled for Friday, June 10 at 7:30 P.M. preceded by cocktails at 6:30 P.M.

All members are urged to make their hotel reservations immediately. The Eden Roc will hold 250 bedrooms and the adjoining hotels, the Montmartre Hotel and Lucerne Hotel will hold 75 rooms each, for members until May 1, 1960, after which time the hotel will not guarantee further reservations. Therefore it is imperative that you make your reservations now directly with the hotel, advising them of time and date of arrival and departure. If you plan on remaining through June 12th for the American Medical Association and other meetings, it is imperative that your reservation be made from June 12th on, directly through the A.M.A. Housing Bureau, c/o Miami Beach Convention Bureau, 1700 Washington Avenue, Miami Beach, Florida, since no reservation beyond this date can be accepted directly by the hotels. Make your reservations now and avoid disappointment. Hotel reservation card will be mailed to members shortly after January 1, 1960.

Final program, membership card and advance registration forms will be sent on May 1st 1960 to members whose current dues have been paid.

1960 AWARDS

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

Fred Conrad Koch Award

During the past year a substantial legacy has been bequeathed the Society by the late Elizabeth Koch for the purpose of establishing the Fred Conrad Koch Memorial Fund in memory of her late husband, distinguished service professor of physiological chemistry at the University of Chicago and pioneer in the isolation of the androgens. This is to be the highest honor of the Endocrine Society and is to be represented by a medal that is to be known as the Koch Medal of The Endocrine Society. The medal and honorarium of $3,500 is to be given annually to an individual for work of special distinction in endocrinology.

The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. This award will replace the Medal of the Endocrine Society which was established in 1954 and presented to Dr. Carl Moore in 1955, Dr. Frederick L. Hisaw in 1956, Dr. Joseph C. Aub in 1957, Dr. I.L. Chaikoff in 1958 and Dr. Wilbur W. Swingle in 1959. The Endocrine Medal replaced the E. R. Squibb Award which was formerly the highest honor bestowed by the Society. Past recipients of the Squibb Award were Dr. George W. Corner in 1940, Dr. Philip E. Smith in 1941, Dr. Fred C. Koch, 1942, no award was given in 1943, Dr. E. A. Doisy in 1944, Dr. E. C. Kendall in 1945, Dr. Carl O. Hartman in 1946, Drs. Carl F. and Gerty T. Cori in 1947, Dr. Fuller Albright in 1948, Dr. Herbert Evans in 1949, Dr. C. N. H. Long in 1950, Dr. J. B. Collip in 1951, Dr. James H. Means in 1952 and Dr. David Marine in 1953.

The Ciba Award

The Ciba Award, to recognize the meritorious accomplishments of an investigator who has not attained his 36th birthday by June 1st of the year the award is presented, in the field of clinical or preclinical endocrinology, was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood, 1945 Dr. Jane A. Russell, 1946 Dr. Martin M. Hoffman, 1947 Dr. Choh Hao Li, 1948 Dr. Carl Heller, 1949 Dr. George Sayers, 1950 Dr. Oscar M. Hechter, 1951 Dr. Albert Segaloff, 1952 Dr. Seymour Lieberman, 1953 Dr. Sidney Roberts and Dr. Clara Szego (Mrs. Roberts), 1954 Dr. Isadore M. Rosenberg, 1955 Dr. Jack Gross, 1956 Dr. Alfred M. Bongiovanni, 1957 Dr. Nicholas S. Halmi, 1958 Dr. Monte Arnold Greer, and 1959 Dr. Gordon L. Farrell. Prior to 1952 the Award was $1,200. It has now been increased to $2,500.

The Ayerst and the Squibb Fellowships

The Ayerst Fellowship was established in 1947 and the Squibb Fellowship in 1956. They are designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology.

Each Fellowship is awarded on alternate years and the stipend, which will not exceed $5,000 may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree, or candidates for either of these degrees, are eligible for appointment.

Applicants must submit the following information:

1. Evidence of scientific ability as attested by studies completed or in progress.
2. Recommendations from individuals familiar with the candidate and his work.
3. A proposed program of study.
4. Acceptance of the individual by the head of the department in which the Fellowship will be held.
5. A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Schering and the Upjohn Scholars of The Endocrine Society

The Council of The Endocrine Society has established a category of Scholars. These grants have been made available through the generosity of the Schering Corporation and the Upjohn Company, and will be awarded to established investigators and teachers in the field of endocrinology who wish to extend their opportunities for work either in this country or abroad.

The awards will not exceed $2,500 annually for each individual and will be granted on the basis of proposals submitted by the applicant. Such applications should include estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Fred Conrad Koch Award; the Ciba Award; and the Ayerst and the Squibb Fellowships may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 North Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than October 15, 1959.

Proposals for appointments as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the secretary of the Society. They should be submitted by October 15, 1959.

The Awards Committee will meet in October and notice of awards to successful nominees and applicants will be made not later than December 1st.